NADPH–Cytochrome P-450 Oxidoreductase: Flavin Mononucleotide and Flavin Adenine Dinucleotide Domains Evolved from Different Flavoproteins†

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ABSTRACT: The FMN-binding domain of NADPH–cytochrome P-450 oxidoreductase, residues 77–228, is homologous with bacterial flavodoxins, while the FAD-binding domain, residues 267–678, shows a high degree of similarity to two FAD-containing proteins, ferredoxin–$NADP^+$ reductase and NADH–cytochrome b_5 reductase. Comparison of these proteins to glutathione reductase, a flavoprotein whose three-dimensional structure is known, has permitted tentative identification of FAD- and cofactor-binding residues in these proteins. The remarkable conservation of sequence between NADPH–cytochrome P-450 oxidoreductase and ferredoxin–$NADP^+$ reductase, coupled with the homology of the FMN-binding domain of the oxidoreductase with the bacterial flavodoxins, implies that NADPH–cytochrome P-450 oxidoreductase arose as a result of fusion of the ancestral genes for these two functionally linked flavoproteins.

NADPH–cytochrome P-450 oxidoreductase (P450R)[1] is a 78 225-dalton flavoprotein containing 1 mol each of FAD and FMN (Iyanagi & Mason, 1973). This protein is bound to the endoplasmic reticulum (Williams & Kamin, 1962; Phillips & Langdon, 1962) and nuclear envelope (Kasper, 1971) of a variety of cell types and is responsible for the transfer of reducing equivalents to the cytochromes P-450 (Lu et al., 1969) as well as other microsomal enzyme systems, including heme oxygenase (Schacter et al., 1972) and the fatty acid desaturation (Enoch & Strittmatter, 1979) and elongation (Ilan et al., 1981) systems. Electron transfer proceeds from NADPH to FAD to FMN to cytochrome P-450 or another electron-acceptor protein (Vermilion et al., 1981). We have previously reported the amino acid sequence of rat liver P450R, determined from cloned cDNAs to rat liver P450R mRNA (Porter & Kasper, 1985). Residues 77–228 are homologous with the bacterial flavodoxins, indicating that this segment of P450R is likely to bind FMN. Here we expand on the characterization of the FMN domain and report that in the region defined by residues 267–678 of P450R, a high degree of similarity exists with two other FAD-containing proteins, ferredoxin–$NADP^+$ reductase from the blue-green algae *Spirulina* (Yao et al., 1984) and NADH-cytochrome b_5 reductase from human erythrocytes (Yubisui et al., 1984). Comparison of these proteins to glutathione reductase, a flavoprotein whose three-dimensional structure is known (Schulz et al., 1978; Thieme et al., 1981), has permitted tentative identification of FAD- and cofactor-binding residues in these proteins. The evidence presented indicates that the cytochrome P-450 oxidoreductase gene arose through fusion of the ancestral genes of flavodoxin and ferredoxin–$NADP^+$ reductase.

FMN Domain. Graphic matrix computer programs (Maizel & Lenk, 1981) were used to identify regions of similarity between P450R and other flavoproteins. The computer-generated dot matrix comparison of P450R and *Desulfovibrio vulgaris* flavodoxin shown in Figure 1A illustrates the surprising degree of similarity between the amino-terminal portion of P450R, residues 90–210, and flavodoxin. This similarity is clearly defined in the sequence alignment of P450R with flavodoxins from four bacterial species (Figure 2). Three segments within this alignment show particularly high levels of similarity and correspond to regions of the flavodoxin molecule directly involved in binding the FMN prosthetic group. The first segment, which corresponds to P450R residues 84–95, encompasses the FMN phosphate-binding residues (indicated with asterisks). Three of the five residues, Ser-10, Thr-12, and Thr-15 of *D. vulgaris* flavodoxin, hydrogen bond the FMN phosphate oxygens through their side-chain hydroxyl groups, while hydrogen bonding through the polypeptide backbone of Thr-11 and Asn-14 is also involved (Watenpaugh et al., 1973). Amino acids that make hydrogen bonds through their side chains would be expected to be under greater evolutionary constraint than those that make main-chain hydrogens bonds; this is seen in the FMN phosphate-binding segment, where Ser-10 and Thr-12 (side-chain bonding) are conserved in all five proteins, and Thr-15 (also side-chain bonding) is conserved in four out of five proteins. In contrast, Asn-14 (main-chain bonding) is conserved in only three of the proteins, and Thr-11 (also main-chain bonding) is not conserved at all. Notably, in P450R the *number* of residues capable of making side-chain hydroxyl bonds with the FMN phosphate group is the same as for the flavodoxins, 3, with Thr-90 replacing Asn-14, while Ala-91 replaces Thr-15 of *D. vulgaris* flavodoxin.

The remaining two segments of strong sequence conservation between P450R and the flavodoxins correspond to regions of the flavodoxin molecule which interact with the flavin isoalloxazine ring. X-ray crystallographic studies on the flavodoxins from *D. vulgaris* and *Clostridium MP* show that the flavin is positioned between Trp-60 and Tyr-98 in the D. *vulgaris* protein (Watenpaugh et al., 1973) and between Met-56 and Trp-90 in the clostridial protein (Burnett et al., 1974). Both sets of residues occupy approximately equivalent positions in the sequence alignment of Figure 2; the *D. vulgaris* positions are indicated with asterisks. The position facing the interior of the protein, corresponding to Trp-60 and Met-56, is held by hydrophobic residues in all five flavoproteins (Tyr-140 or P450R), consistent with its role in shielding and stabilizing the isoalloxazine ring. It is preceded by a short stretch of hydrophobic residues in all five proteins (Leu-132 to Phe-135 of P450R) and followed immediately by a strongly

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[1] Abbreviations: P450R, NADP–cytochrome P-450 oxidoreductase; FNR, ferredoxin–$NADP^+$ reductase; b_5R, NADH–cytochrome b_5 reductase; GR, glutathione reductase.

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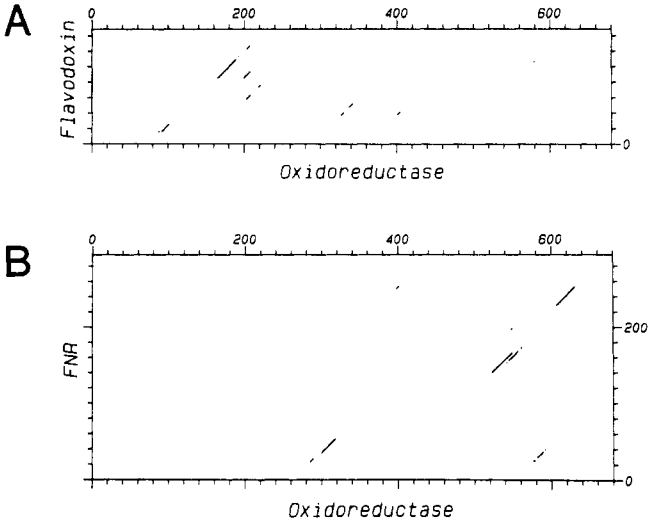

FIGURE 1: Dot matrix comparisons. The two protein sequences indicated on the *x* and *y* axes were compared in all possible registers by using the Compare Program (Maizel & Lenk, 1981) of the University of Wisconsin Genetics Computer Group. Amino acid matches are scored 1.0 and mismatches 0, with a window of 30 residues and a match stringency of 8.0. A point is plotted at the center of the window each time the match stringency is met. (A) Comparison of *Desulfovibrio vulgaris* flavodoxin (Dubourdieu & Fox, 1977) and NADPH–cytochrome P-450 oxidoreductase. (B) Comparison of *Spirulina* ferredoxin–$NADP^+$ reductase (FNR) and NADPH–cytochrome P-450 oxidoreductase.

conserved glycyl residue and then a series of acidic amino acids. Notably, it recently has been suggested (Weber & Tollin, 1985) that these acidic residues are involved in electrostatic charge pairing during the formation of electron-transfer complexes between flavodoxin and *c*-type cytochromes. The presence of acidic amino acids in P450R in this region (i.e., Glu-142, Asp-144, and Asp-147) suggests that these residues may be involved in charge pairing between P450R and the cytochromes P-450.

The exterior flavin-shielding residue of *D. vulgaris* flavodoxin, Tyr-98, is conserved in all five flavoproteins (indicated with an asterisk). In *D. vulgaris*, this tyrosine is coplanar with the flavin, permitting stacking interactions between these two aromatic groups. Despite the conservation of this tyrosine in the clostridial protein (Tyr-88), X-ray crystallographic studies have shown that the tryptophan at position 90 is the exterior flavin-shielding residue in this protein (Burnett et al., 1974). This two amino acid shift of the second flavin-shielding residue toward the carboxy terminus may be a compensatory response to the overall shift of sequence toward the amino terminus, as reflected by the gap in the clostridial sequence in the region preceding the conserved tyrosyl residue. The flavodoxin from *Megasphaera elsdenii* resembles the clostridial protein in this respect. Because the P450R sequence most closely resembles *D. vulgaris* flavodoxin, it seems probable that Tyr-178 of P450R serves as the second flavin-shielding residue in this protein.

The predicted secondary structure of the FMN domain of P450R can be seen in the upper portion of Figure 3. Perhaps not surprisingly, the predicted structure is very similar to that determined by X-ray crystallography of *D. vulgaris* flavodoxin (Watenpaugh et al., 1973). Notably, the phosphate-binding residues, 86–91, are located in a loop between a β-strand and a following α-helix, and the two flavin-shielding residues are located at or near the carboxyl ends of β-strand structures. The strong similarity in both sequence and secondary structure of P450R and flavodoxin indicates that the FMN domain of P450R is likely to have a three-dimensional structure very similar to that of flavodoxin, despite its incorporation into a much larger protein.

FAD Domain. Ferredoxin–$NADP^+$ reductase (FNR) is a FAD-containing protein that catalyzes electron transfer from

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                                  * * *  * *
P450R          77  ..GRNIIVFY GSQTGTAEEF ANRLSKDAHR YGMRGMSADP 114
D. vulgaris     1    MPKALIVY GSTTGNTEYT AETIARELAN AGYEVDSRDA  38
M. elsdenii     1      MVEIVY WSGTGNTEAM ANEIEAAVKA AGADVESVRF  36
Clostrd. MP     1       MKIVY WSGTGNTEKM AELIAKGIIE SGKDVNTINV  35
Azoto. vin.     1     AKIGLFF GSNTGKTRKV AKSIKKRFDD ETMSDALNVN  37

                                    *
115  EEYDLADLSS LPEIDKSLVV FCMATYGEGD PT......DN AQDFYDWLQE 158
 39  ASVEAGGL.. ..FEGFDLVL LGCSTWGDDS IE......LQ DDFIPLFDSL  78
 37  EDTNVDD... ..VASKDVIL LGCPAMGSEE LE........ DSVVEPFFTD  73
 36  SDVNIDE... ..LLNEDILI LGCSAMGDEV LE........ ESEFEPFIEE  72
 38  RVSAED.... ..FAQYQFLI LGTPTLGEGE LPGLSSDCEN ESWEEFLPKI  81

                                    *
159  TDVDLTGVKF AVFGLGNK.T YEHFNAMGKY VDQRLEQLGA QRIFELGLGD 207
 79  EETGAQGRKV ACFGCGDS.S YEYFCGAVDA IEEKLKNLGA EIVQDGLRID 127
 74  LAPKLKGKKV GLFG.....S YGWGSGEWMD AWKQRTEDTG ATVIGTAIVN 118
 73  ISTKISGKKV ALFG.....S YGWGDGKWMR DFEERMNGYG CVVVETPLIV 117
 82  EGLDFSGKTV ALFGLGDQVG YPENYLDALG ELYSFFKDRG AKIVGSWSTD 131

208  DDGNLEEDFI TWREQFWPAV C...                          228
128  GDPRAARDDI VGWAHDVRGA I                              148
119  EMPDNAPECK ELGEAAAKA                                 137
118  QNEPDEAEQD CIEFGKKIAN I                              138
132  GYEFESSEAV VDGKFVGLAL D...                           152
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FIGURE 2: Alignment of NADPH–cytochrome P-450 oxidoreductase (P450R) with flavodoxins from *Desulfovibrio vulgaris*, *Magasphaera elsdenii* (Tanaka et al., 1973, 1974a), *Clostridium MP* (Tanaka et al., 1974b), and *Azotobacter vinelandii* (Tanaka et al., 1977). The sequences were aligned by using the alignment algorithm of Needleman and Wunsch (1970). Final alignments were obtained by visual inspection, taking into account the amino acid comparison values from the relatedness odds matrix of Dayhoff (1978). Gaps inserted in the sequence are indicated by dots. The left and right amino acids of each line are numbered. Identical amino acids are boxed; the positions indicated with asterisks are discussed in the text.

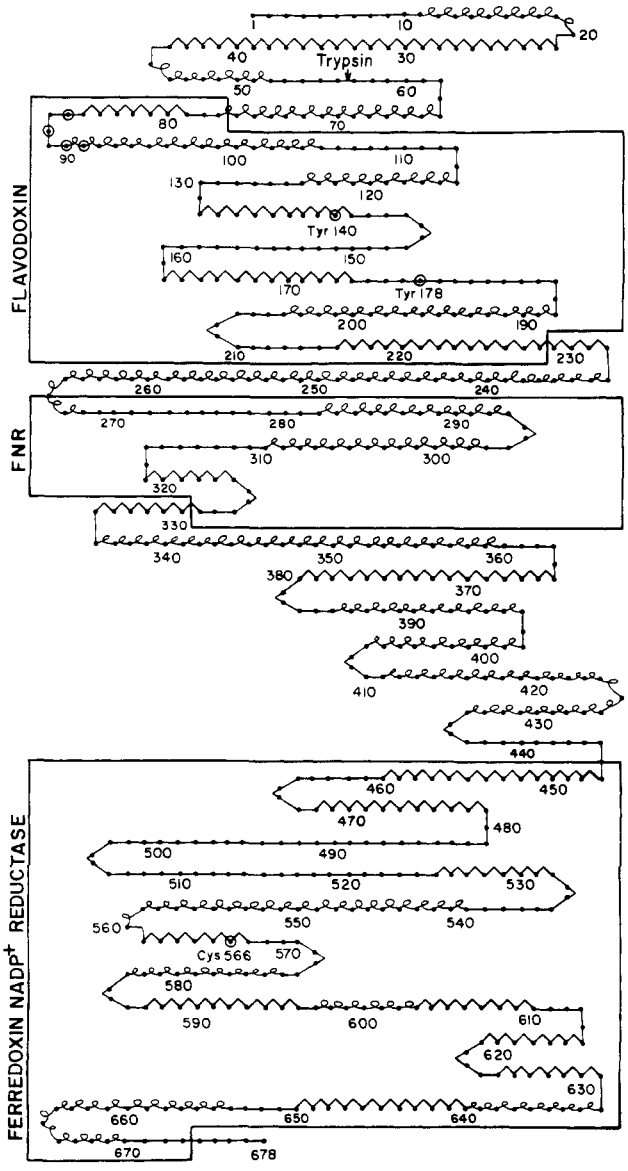

FIGURE 3: Predicted secondary structure of NADPH–cytochrome P-450 oxidoreductase as determined by the method of Chou and Fasman (1978). Loops indicate α-helix; zig-zags, β-strand; straight lines, random coil. Predicted β-turns are depicted as sharply angled reverses, as shown between residues 144 and 147. The uniquely sensitive, trypsin-cleavable Lys-56–Ile-57 bond demarcating the membrane-binding segment from the catalytic portion of the enzyme is indicated. Segments homologous with flavodoxin and ferredoxin–$NADP^+$ reductase (FNR) are boxed. Tyr-140 and -178, the predicted FMN isoalloxazine ring-flanking residues, are circled, as are the predicted FMN phosphate-binding residues 86, 88, 90, and 91. The NADPH-protectable cysteinyl residue, Cys-566, is also circled.

reduced ferredoxin to $NADP^+$ during photosynthesis (Zanetti & Forti, 1966). Comparison of P450R to FNR (Figure 1B) reveals several stretches of similarity occurring in the carboxy-terminal portion of P450R. These results suggest that, while the amino-terminal portion of P450R shares a common ancestry with flavodoxin, the carboxy-terminal, FAD-containing portion of the protein may have evolved from a second type of ancestral flavoprotein, distinct from flavodoxin, to which FNR is also related. The initial match to FNR includes P450R residues 280–320 and corresponds to FNR residues 20–55. This match is approximately 120 amino acids out of register with the remaining two matches, shown spanning P450R from residues 520 to residue 630 (FNR residues 140–255). Careful alignment of the two protein sequences indicates that this shift in register is probably due to an insertion in the P450R sequence, as discussed below.

Figure 4 shows the alignment of the carboxy-terminal segment of P450R, residues 267–678, with the complete sequence of FNR. The regions of similarity suggested in Figure 1B between these two flavoproteins can be identified by direct comparison of the two sequences. Also shown in Figure 4 is an alignment of the NADH–cytochrome b_5 reductase (b_5R) sequence with the P450R and FNR sequences. b_5R is an FAD-containing protein involved in methemoglobin reduction in erythrocytes (Kuma & Inomata, 1972); there is also a liver microsomal form, which is bound to the endoplasmic reticulum by a hydrophobic amino-terminal segment (Kensil et al., 1983) not present in the erythrocytic form. The considerable similarity between P450R, FNR, and b_5R evident from Figure 4 suggests that all three flavoproteins evolved from a common ancestral protein and are likely to be structurally similar. We have attempted to identify segments involved in flavin and pyridine nucleotide cofactor binding in these proteins by sequence comparison to glutathione reductase (GR), a flavoprotein whose sequence (Krauth-Siegel et al., 1982) and three-dimensional structure (Thieme et al., 1981) are known. GR is an FAD-containing enzyme that uses NADPH as a source of reducing equivalents. The relationship of the peptide chain to the binding of FAD and NADPH has been extensively characterized (Schulz et al., 1978, 1982; Thieme et al., 1981; Rice et al., 1984), making GR the best characterized FAD-containing protein to date and an appealing model for comparison to other flavoproteins.

Residues 24–58 of GR encompass the highly conserved FAD pyrophosphate-binding segment of this protein (Schulz et al., 1982). Dot matrix comparisons indicated that this segment could be aligned with the P450R, FNR, and b_5R sequences as shown in the upper portion of Figure 4, designated "FAD–PP_i binding". Although the number of amino acid matches that P450R, FNR, and b_5R have in common with GR in this segment is limited, when dot matrix comparisons are run by using the amino acid comparison values of Dayhoff (1978), which allow for substitutions between similar amino acids, the similarity between these proteins in this segment becomes more evident. In GR, the three glycyl residues indicated with asterisks (Gly-27, -29, and -32) are in direct contact with the pyrophosphoryl portion of the FAD molecule and are strongly conserved in a number of FAD-containing proteins (Schulz et al., 1982; Rice et al., 1984). However, in the alignment of Figure 4 only the first of these glycines is conserved, and only in P450R and FNR. In GR, these residues fall in a tight loop between the first β-strand and the following α-helix of a Rossmann fold structure (Schulz et al., 1982). Interestingly, the predicted secondary structure of this region of P450R by the method of Chou and Fasman (1978), as shown in Figure 3, indicates that this segment adopts a modified Rossmann fold structure, where the first β-strand is instead replaced by an α-helix (residues 282–292). Like GR, however, the proposed pyrophosphate-binding residues are located in a loop between this and the following α-helix (residues 293–296). This similarity in secondary structure between GR and P450R, where the proposed pyrophosphate-binding residues of P450R are located in an unstructured segment between two structural regions, as is seen in GR, is consistent with this region of P450R (and FNR and b_5R) being adjacent to the FAD pyrophosphate group. It is noteworthy that this is the first example of a group of FAD-containing proteins that do not conform to the "Gly-X-Gly-X-X-Gly rule" for pyrophosphate-binding segments. In this same stretch of residues, Glu-50 of GR, also indicated with an asterisk, binds the ad-

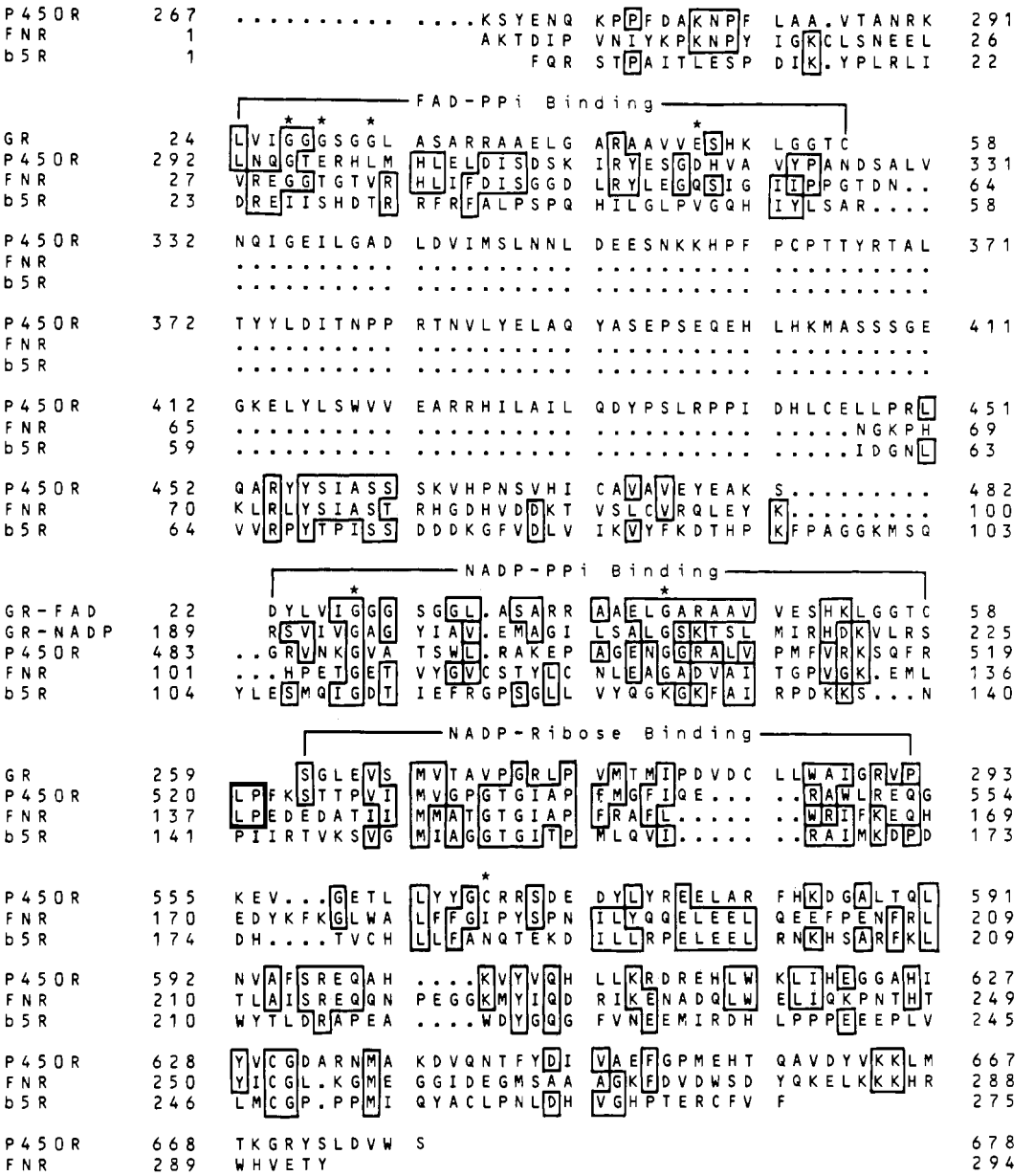

FIGURE 4: Alignment of NADPH–cytochrome P-450 oxidoreductase (P450R), ferredoxin–$NADP^+$ reductase (FNR), and NADH–cytochrome b_5 reductase (b_5R). The amino acid sequences of P450R, FNR, and b_5R were compared to each other and to the sequence of glutathione reductase (GR) with the Compare Program, and regions of similarity were aligned by using the alignment algorithm of Needleman and Wunsch (1970). The final alignments were obtained by visual inspection, taking into account the amino acid comparison values from the relatedness odds matrix of Dayhoff (1978). Gaps inserted in the sequences are indicated by dots. The left and right amino acids of each line are numbered. GR–FAD indicates the proposed GR–FAD pyrophosphate binding segment; GR–NADP indicates the proposed GR–$NADP^+$ pyrophosphate binding segment. Identical residues are boxed; the positions indicated with asterisks are discussed in the text.

enine ribose hydroxyl groups of the FAD molecule (Schulz et al., 1982). Although, as shown by Rice et al. (1984), this residue is conserved among other FAD-containing proteins, including lipoamide dehydrogenases, *p*-hydroxybenzoate hydroxylase, and mercuric reductase, it is replaced by aspartate in P450R, glutamine in FNR, and valine in b_5R. Although the first two substitutions would appear to be functionally conserved, the substitution of valine in this position, coupled with the lesser similarity overall between b_5R and P450R, FNR, and GR in this segment, suggests that the tertiary structure of b_5R may differ significantly from the tertiary structure of these flavoproteins in this region.

Following the proposed FAD pyrophosphate-binding segment of these proteins is a segment of 117 amino acids in P450R that has no equivalent match in FNR or b_5R (as suggested from the dot matrix comparison of P450R and FNR, Figure 1B). Because P450R contains an FMN prosthetic group, in addition to FAD, the enzyme is likely to possess structural features necessary to couple electron transfer between the two flavins. These features would not be found in either FNR or b_5R, where electron transfer is strictly intermolecular. Although the mechanism of electron transfer in P450R is unknown, it is possible that this 117 amino acid "insertion", relative to FNR and b_5R, is involved in coupling the two flavin domains. Following this insertion is a short segment of highly conserved residues, extending from Arg-454 to Ser-461 of P450R. The high degree of conservation of this segment in P450R, FNR, and b_5R suggests that it may have a specific functional role. By analogy with GR, this portion of the polypeptide chain would be in a position to interact with the FAD isoalloxazine ring, although no sequence similarity with GR can be discerned in this region. This was also recently suggested by Karplus et al. (1984) on the basis of a comparison of spinach and *Spirulina* FNR sequences.

It is thought that the $NADP^+$ domain and FAD domain of GR arose through a gene duplication event, as the two domains have similar three-dimensional structures (Schulz, 1980). However, very little sequence similarity remains, since the two domains have diverged to perform different functions. One region where some sequence simiarity can still be observed is between the FAD and NADPH pyrophosphate-binding segments. These segments are aligned with segments of P450R, FNR, and b_5R as shown in Figure 4 under "NADP–PP_i binding". In GR, the pyrophosphoryl group of $NADP^+$ is hydrogen bonded to three sequential residues, Gly-194, Ala-195, and Gly-196 (Krauth-Siegel et al., 1982; Rice et al., 1984). Gly-194 is highly conserved in a number of nucleotide-binding proteins, as in Gly-210 (Hofsteenge et al., 1980; Rice et al., 1984); both residues are indicated with asterisks. These residues are conserved in P450R (Gly-488 and -504, FNR (Gly-105 and -122), and b_5R (Gly-111 and -127 or -129), suggesting that this region of these proteins is involved in binding the pyrophosphoryl portion of $NAD(P)^+$. The 2′-phosphate of the $NADP^+$ ribose group is hydrogen bonded to Arg-218, His-219, and Arg-224 in GR (Krauth-Siegel et al., 1982; Rice et al., 1984); hence, these residues appear to be important for the selective binding of NADPH over NADH. It would be expected that these or equivalent residues would be conserved in P450R and FNR, but this does not appear to be the case; indeed, the substitution of hydrophobic residues (Phe, Val, Met) in these positions in P450R and FNR suggests that the specificity for NADP(H) as a cofactor must reside elsewhere in these two enzymes. Rice et al. (1984) have suggested that NAD^+ binding in lipoamide dehydrogenase, an enzyme structurally similar and evolutionarily related to GR, is specified by a glutamyl residue (Glu-204) which would hydrogen bond the 2′- and 3′-ribose hydroxyl groups of NAD^+. A similar orientation is found in other NAD^+-requiring dehydrogenases, where an aspartyl residue binds the NAD^+ ribose hydroxyls (Rossmann et al., 1975). The alignment of the b_5R sequence suggests that the aspartate at position 136 performs this function in this enzyme.

A final section of similarity between these four proteins immediately follows the $NADP^+$ pyrophosphate binding region and corresponds to a segment of GR that lies in close proximity to the adenine ribosyl portion of the $NADP^+$ molecule (Rice et al., 1984). This segment, identified in Figure 4 as "NADP–ribose binding", encompasses residues 259–293 of GR. A surprising amount of sequence conservation can be noted between all four proteins in this segment, including the positions corresponding in GR to Met-265, Gly-271, and Pro-274. This high degree of conservation suggests that these residues are important in recognizing and binding the nucleotide cofactor in all four flavoproteins. Amino acids 288–291 of this GR segment (Ala-Ile-Gly-Arg) comprise a second group of residues that form main-chain hydrogen bonds with the pyrophosphoryl portion of $NADP^+$ (Rice et al., 1984). The corresponding residues in P450R, FNR, and b_5R are either conserved or represent conservative substitutions, suggesting that this segment of these proteins also interacts with the $NAD(P)^+$ pyrophosphoryl group. The several basic amino acids present in this segment (i.e., Arg-547 and -551 in P450R) could then charge complement the negatively charged pyrophosphate.

On the basis of the above sequence similarities, the overall arrangements of the structural regions of P450R, FNR, and b_5R show a remarkable degree of similarity to the structural organization of GR, suggesting that all four flavoproteins are homologous. With GR as a model, the remaining 100 or so amino acids of P450R, FNR, and b_5R between the NADP–ribose-binding segment and the carboxy termini are likely to fold back toward the FAD group so as to situate the nicotinamide ring of the appropriate cofactor in a position that will permit electron transfer between it and the flavin isoalloxazine ring. Because the carboxy-terminal portion of GR is specialized to bind oxidized glutathione and catalyze its reduction, it would not be expected to show sequence similarity with P450R, FNR, and b_5R in this region. Nonetheless, in this region (P450R residues 555–678) considerable sequence similarity exists between P450R, FNR, and b_5R; this conservation of sequence between these three flavoproteins argues that this region is involved in cofactor binding. Preliminary results from X-ray crystallographic studies of FNR (Sheriff & Herriott, 1981) support this hypothesis. Finally, Haniu et al. (1984) recently sequenced a tryptic peptide from porcine P450R which contained a cysteinyl residue that, in the intact protein, could be protected from thiol-specific reagents by bound NADPH. This peptide, which corresponds to rat P450R residues 556–567 (Cys-566, indicated with an asterisk), is found immediately following the NADP–ribose-binding segment, providing physical evidence that this region of P450R, and probably FNR and b_5R, is involved in cofactor binding.

Conclusions

The amino-terminal homology of P450R with flavodoxin, and the carboxy-terminal homology with FNR, b_5R, and GR, demonstrated here poses an interesting question as to the evolutionary origin of P450R. Flavodoxins constitute a group of low molecular weight, FMN-containing proteins that catalyze electron transfer to and from other redox proteins and are able in many cases to replace ferredoxins in a wide variety of electron-transport reactions. They have been found in bacteria, blue-green algae, and a green alga, but not yet in higher plants and animals. Of particular interest here is the demonstrated ability of flavodoxin to replace ferredoxin in the light-dependent reduction of $NADP^+$ by FNR (Smillie, 1965; Knight & Hardy, 1967). The homology of the amino-terminal portion of P450R with flavodoxin, and the carboxy-terminal homology with FNR, suggests that P450R arose through a fusion of the ancestral genes for these two functionally linked flavoproteins. It seems reasonable to hypothesize that the genes for flavodoxin and FNR may have been arranged in tandem in an operon of an early organism and at some point fused to give rise to a P450R precursor. In the present enzyme, approximately 40 amino acids separate the FMN (flavodoxin) and FAD (FNR) domains (see Figure 3); the origin of this short segment is unclear, but it may have been present in one or the other precursor proteins, or it may have evolved sometime thereafter to enhance the coupling of the two flavin domains. Interestingly, this stretch of residues is predicted to be almost entirely α-helix, with a predominance of negatively charged residues. An interesting question is how electron transfer is effected between the two domains; it seems unlikely that simply coupling flavodoxin and FNR in a "head to tail" orientation would correctly position the two proteins for effective electron transfer. We suggest that the large insertion in the P450R sequence, residues 330–446, may be required for proper positioning of the FMN and FAD domains so as to permit efficient electron transfer. This insertion may also represent a portion of P450R that specifically interacts with the cytochromes P-450. Elucidation of the precise mechanism of electron transfer between the flavins will be aided significantly by structural information gained from X-ray crystallographic analysis; determination of the distribution of exons within the P450R gene, currently under way in our laboratory,

may provide support for the gene fusion hypothesis proposed here.

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Registry No. P450R, 9039-06-9; b_5R, 9032-25-1; FNR, 9029-33-8; FAD, 146-14-5; FMN, 146-17-8; GR, 9001-48-3.

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