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^{31}P NMR Spectroscopic Studies on Purified, Native and Cloned, Expressed Forms of NADPH–Cytochrome P450 Reductase†

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ABSTRACT: ^{31}P NMR spectroscopy has been utilized in conjunction with site-directed mutagenesis and phospholipid analysis to determine structural aspects of the prosthetic flavins, FAD and FMN, of NADPH–cytochrome P450 reductase. Comparisons are made among detergent-solubilized and protease (steapsin)-solubilized preparations of porcine liver reductases, showing unequivocally that the ^{31}P NMR signals at ~0.0 ppm in the detergent-solubilized, hydrophobic form are attributable to phospholipids. By extraction and TLC analysis, the phospholipid contents of detergent-solubilized rat liver reductase, both tissue-purified and *Escherichia coli*-expressed, have been determined to reflect the membranes from which the enzyme was extracted. In addition, the cloned, wild-type NADPH–cytochrome P450 reductase exhibits an additional pair of signals downfield of the normal FAD pyrophosphate resonances reported by Otvos et al. [(1986) *Biochemistry 25*, 7220–7228], but these signals are not observed with tissue-purified or mutant enzyme preparations. The $Tyr_{140} \rightarrow Asp_{140}$ mutant, which exhibits only 20% of wild-type activity, displays no gross changes in ^{31}P NMR spectra. However, the $Tyr_{178} \rightarrow Asp_{178}$ mutant, which has no catalytic activity and does not bind FMN, exhibits no FMN ^{31}P NMR signal and a normal, but low intensity, pair of signals for FAD. The latter experiments, taking advantage of mutations in residues putatively on either side of the FMN isoalloxazine ring, suggest subtle to severe changes in the binding of the flavin prosthetic groups and, perhaps, cooperative interactions of flavin binding to NADPH–cytochrome P450 reductase.

Microsomal NADPH–cytochrome P450 reductase (EC 1.6.2.4) is one of only two mammalian enzymes known to contain both FAD and FMN (Iyanagi & Mason, 1973; Masters et al., 1975; Dignam & Strobel, 1975), the other enzyme being nitric oxide synthase from rat cerebellum (Bredt & Snyder, 1990; Bredt et al., 1991). NADPH–cytochrome P450 reductase (hereafter referred to as reductase) is a component of the cytochrome P450 monooxygenase system which is responsible for the oxidative metabolism of various xenobiotics, as well as many endogenous compounds (Conney, 1967; Gillette et al., 1972; Masters & Okita, 1980). The reductase is also capable of transferring reducing equivalents to several other heme proteins, such as heme oxygenase (Schacter et al., 1972; Yoshida, et al., 1980), cytochrome *c* (Horecker, 1950), and cytochrome b_5 (Enoch & Strittmatter, 1979). The major function of the reductase is to transfer electrons provided by NADPH to the heme iron of the cytochrome P450 molecule. The electron-transfer sequence is known to occur in the following manner (Vermilion et al., 1981):

$$\text{NADPH} \rightarrow [\text{FAD} \rightarrow \text{FMN}] \rightarrow \text{cytochrome P450}$$

Reductase has been purified from several species with reported molecular masses varying between 76 and 81 kDa (Masters, 1980). The amino acid sequence has been determined for several of the reductases, such as rat (Porter & Kasper, 1985), rabbit (Katagiri et al., 1986), pig (Haniu et al., 1986; Vogel & Lumper, 1986), yeast (Yabusaki et al., 1988; Sutter et al., 1990), trout (Urenjak et al., 1987), and human (Yamano et al., 1989; Haniu et al., 1989). Very high sequence homology exists among the various reductases, consistent with the im-

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portance of the enzyme in the course of evolution. The enzyme has two structural (hydrophilic and hydrophobic) domains and several functional domains (Porter & Kasper, 1986). The hydrophobic N-terminus serves to anchor the protein molecule to the endoplasmic reticulum (ER) and nuclear envelope (Kasper, 1971), thus ensuring proper spatial interaction for electron transfer between the reductase and cytochrome P450. The hydrophilic domain is about 70 kDa and has the specific regions necessary to bind the flavin cofactors, NADPH, and, presumably, cytochrome *c* and cytochrome P450. Digestion with pancreatic steapsin or trypsin releases the C-terminal domain from the lipid bilayer in soluble form. The steapsin-solubilized reductase (Williams & Kamin, 1962), while retaining the ability to transfer electrons to artificial electron acceptors, is incapable of reducing cytochrome P450 (Black & Coon, 1982). The reductase can also be solubilized with any of several commercially available detergents and purified by biospecific affinity chromatography (Yasukochi & Masters, 1976; Strobel & Dignam, 1978). Reductase prepared in this fashion can be added to various microsomal cytochromes P450, in the presence of phospholipids, to reconstitute the monooxygenation of a variety of substrates, the specificities for which are determined by the cytochrome P450 utilized.

A number of biophysical techniques have been employed in the past to study the conformational characteristics of the reductase molecule in solution, and to understand its physical and electronic interactions with NADPH, the flavin prosthetic groups, and cytochrome P450. Methods such as ESR spectroscopy (Iyanagi & Mason, 1973; Yasukochi et al., 1979), time-resolved fluorescence spectroscopy (Blumberg et al., 1982; Bastiaens et al., 1989), ^{31}P NMR spectroscopy (Otvos et al., 1986; Bonants et al., 1990), and resonance Raman spectroscopy (Lively et al., 1984; Sugiyama et al., 1985) have yielded some useful information in this regard, but X-ray diffraction techniques (Sugiyama et al., 1983) have been thwarted by the nonavailability of crystals of diffraction quality and size. Our laboratories have shown that ^{31}P NMR spectroscopic methods can be successfully applied to the study of the steapsin-solubilized porcine reductase (Otvos et al., 1986; Masters et al., 1990; Narayanasami et al., 1991). All of the phosphorus-containing cofactors in the porcine liver enzyme were found to give rise to well-resolved ^{31}P NMR resonances which could be assigned individually to FAD or FMN (or to phosphorus-containing compounds adventitiously bound to the reductase). By monitoring the influence of the paramagnetic center in the air-stable semiquinone form of the enzyme on the line widths of these signals, it was established that the unpaired electron is localized on the FMN rather than the FAD moiety (Otvos et al., 1986).

Recently, Shen et al. (1989) have created several single- and double-site mutants in which the Tyr residues at positions 140 and 178, implicated in the binding of FMN, were replaced with Asp and Phe. These researchers have also determined the various kinetic parameters as well as flavin contents of these mutants in comparison to the wild-type and the tissue-purified forms. We thought it would be very informative to employ ^{31}P NMR spectroscopic techniques to determine the effects of altering the nature of interaction between the key FMN-binding residues, Tyr_{140} and Tyr_{178} and the flavins.

The present work extends our previous studies through the examination of the ^{31}P NMR characteristics of detergent-solubilized porcine, rat, and cloned rat liver (wild-type and mutant) reductase preparations and porcine liver reductase solubilized with pancreatic steapsin. The effects of manganese on the ^{31}P NMR signals have permitted the determination of relative exposure to bulk solvent of the various phosphorus-containing moieties. In addition, ^{31}P NMR signals attributable to phospholipid (PL) were identified in several detergent-solubilized preparations of the enzyme and in the cloned reductase from *Escherichia coli*. The data thus far indicate that the PL contents of the various preparations represent the membrane environments from which they were extracted, i.e., endoplasmic reticulum or *E. coli*, and do not contribute toward the regulatory or structural aspects of the reductase. Some of these data have appeared in preliminary form (Masters et al., 1990; Narayanasami et al., 1991).

Materials and Methods

Materials

Most of the standard chemicals (Analytical Reagent or HPLC grade) were purchased from Sigma Chemical Co. Exceptions are noted where appropriate in the text. Emulgen 913, used in the enzyme solubilization procedures, was a gift from Kao Chemicals (Tokyo, Japan). Other chemicals used in the ^{31}P NMR experiments were from sources indicated in Otvos et al. (1986).

Methods

Purification of Reductase from Pig and Rat Livers. Porcine livers were obtained from freshly sacrificed animals, and after perfusion with physiological saline, the livers were either utilized immediately for enzyme preparation or frozen in small chunks in liquid nitrogen and stored at −80 °C until ready for use. Perfused rat livers were obtained frozen from Pel-Freez and stored at −80 °C, except in the case of the enzyme preparation used to obtain the NMR spectra in Figure 3. In this particular experiment, the enzyme was purified from phenobarbital-induced rats, using the detergent Renex 690 (Yasukochi & Masters, 1976; Vermilion & Coon, 1978). During the night preceding microsome preparation, the livers (rat or pig) were thawed at 4 °C, and the following morning the livers were allowed to thaw completely in the appropriate homogenization buffer, containing 0.1 M potassium pyrophosphate (pH 7.4). The procedure for the purification of reductase from pig and rat livers, except in the case of the preparation used in Figure 3 as mentioned previously, is the same as in Otvos et al. (1986) with the following minor modifications. The concentration of 2′-AMP used for the elution of the bound reductase from the 2′,5′-ADP–Sepharose 4B (Pharmacia Fine Chemicals) affinity column was increased from 2 to 5 mM in order to obtain a steeper elution profile. In addition, the elution buffer was loaded from the bottom of the column so that the eluant fractions of reductase were collected from the top side of the column. This modification resulted in a more rapid and efficient elution of the reductase. In most reductase preparations, the charcoal–Celite treatment step, used for the purpose of removing bound 2′-AMP from the affinity-purified steapsin-solubilized enzyme (Otvos et al., 1986), was omitted.

Purification of the Cloned Reductase from E. coli. The purification of all the *E. coli*-expressed reductase preparations used in this publication was exactly as described in Shen et al. (1989), employing 2-L Fernbach flasks with shaking for the bacterial growth. Selected fractions of the eluant (for both *E. coli* and mammalian reductase preparations) from the 2′,5′-ADP-Sepharose 4B affinity column were pooled on the basis of enzyme purity, as determined by SDS–PAGE electrophoresis and specific activity measurements. The purified enzyme preparation was routinely concentrated on an Amicon ultrafiltration apparatus using either PM-30 or YM-30 membranes (Amicon Co.) to obtain NMR concentrations, viz.,

0.13–0.25 mM, as well as to change the solvent from affinity buffer to 50 mM Tris (pH 7.7), used for all ^{31}P NMR experiments.

Phospholipid Extraction and Analysis. Enzyme samples were extracted according to the method of Bligh and Dyer (1959). Briefly, the samples were mixed with chloroform and methanol so that the proportions of chloroform/methanol/water were 1:2:0.8, the original sample constituting the water portion. The total volume of the extract was usually about 7 mL. The extract was then partitioned into aqueous and organic phases by the addition of chloroform and water, so that the new ratio of chloroform/methanol/water was 2:2:1.8. Samples were vortexed and centrifuged at 800*g* for 10 min to facilitate phase separation. The lower, chloroform-rich layer, containing the extracted lipids, was isolated, evaporated under nitrogen, and redissolved in 0.2 mL of chloroform/methanol (1:1). Silica gel G thin-layer chromatographic (TLC) plates were washed in a neutral solvent mixture containing chloroform/methanol/water (65:35:6) and allowed to air-dry. The plates were activated by heating at about 150 °C for 20 min and allowed to cool. The phospholipid extracted from the enzyme sample, Triton X-100, and a phospholipid standard mixture containing phosphatidylcholine (PC), lyso-PC (LPC), phosphatidylethanolamine (PE), oleic acid (OA), and methyl oleate (ME) were applied to the TLC plate, allowed to dry, and then developed in the same neutral solvent used earlier. The plates were sprayed with 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) (Jones et al., 1982) and visualized under ultraviolet light. The R_f values of the authentic PL samples were determined to be the following: LPC = 0.11; PC = 0.33; PE = 0.63; OA = 0.75; ME = 0.93. The Triton X-100 sample yielded an R_f value of 0.80. The components of the enzyme extracts were identified by comparison of their R_f values with those of the authentic samples. Relevant fractions were purified from silica, scraped from the TLC plates, by solvent extraction. The phospholipids in the TLC fractions were rechromatographed on TLC to confirm their identities and then quantified by phosphorus analysis [modified procedure of Allen (1940)].

^{31}P NMR Methods. The NMR spectral data were obtained on a Bruker WM-250 at 101.2 MHz, as described by Otvos et al. (1986) (Figures 1–4), a General Electric GN-300 at 121.4 MHz (Figure 5), or a Bruker AM-400 at 162 MHz (Figure 6). All experiments were carried out at 10 °C under full broad-band decoupling conditions. The following instrumental conditions were common to all the experiments: spectral window of 5000 Hz, 8K data points, 45° pulse width (except spectra in Figure 5, where the pulse width was 35°), and 0.82-s acquisition time. Typical data acquisition involved the accumulation of 80000–200000 transients, requiring 18–45 h of instrument time. The data were processed in all cases with a line broadening of 15 Hz, except in Figure 1A where 10-Hz line broadening was used. The application of two slightly different line broadening values when comparing two data sets would not have affected any of our conclusions, considering that the line widths of the ^{31}P NMR signals of interest are of the order of 70–100 Hz. All chemical shifts are reported relative to external 85% phosphoric acid. The sample volume was 1.7–2.0 mL in 10-mm thin-walled NMR tubes (Aldrich Chemicals or Wilmad Glass Co.) containing 15–20% D_2O to provide field frequency lock.

In experiments designed to probe the relative degrees of solvent exposure of the various phosphorus-containing moieties in different reductase preparations, paramagnetic Mn(II) ion (as $MnSO_4$) was added to the NMR samples to give a constant Mn/protein molar ratio of about 1.3. Under these conditions, the resonances of completely solvent-exposed species are paramagnetically broadened beyond detection. Control experiments indicated that addition of up to twice this amount of Mn(II) (Mn/protein = 2.6) had no measurable effect on the magnitude of line broadening observed for partially exposed species.

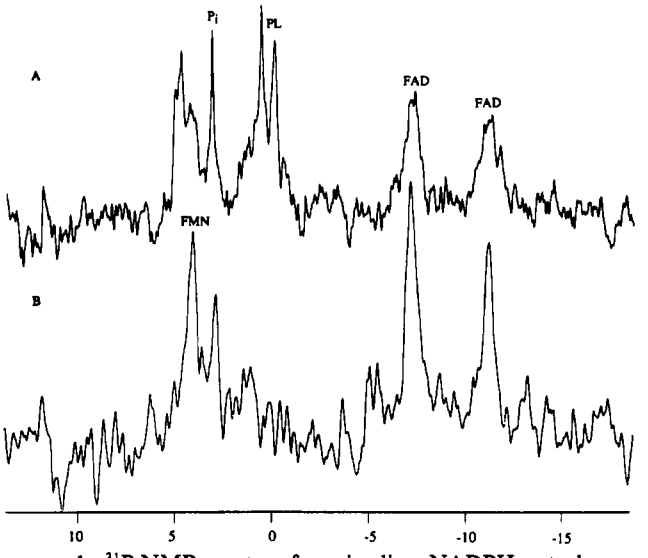

FIGURE 1: ^{31}P NMR spectra of porcine liver NADPH–cytochrome P450 reductase at pH 7.7 at 10 °C: (A) 0.20 mM enzyme solubilized with detergent (Emulgen 913), 120000 transients, line broadening 10 Hz; (B) 0.13 mM enzyme solubilized with pancreatic steapsin, 98000 transients, line broadening 15 Hz. Resonance assignments are based on Otvos et al. (1986).

Results

Steapsin- and Detergent-Solubilized Porcine Liver Reductases. Figure 1 compares the ^{31}P NMR spectra of the steapsin-solubilized (B) and detergent-solubilized (A) porcine liver reductase (DSPR) preparations. The striking difference between the two reductase preparations is the presence of strong signals resonating at 0.4 and −0.3 ppm only in the spectrum of the detergent-solubilized enzyme (Figure 1A). By comparison with published phospholipid (PL) chemical shift data (Sotirhos et al., 1986; Henderson et al., 1974), these signals were tentatively assigned to PL. Upon further examination, the intensities and chemical shifts of the putative PL resonances were found to be quite variable from preparation to preparation. These signal intensities typically vary from undetectable to roughly the magnitude of the flavin signals, while the chemical shifts are in the range of 0.5 to −0.5 ppm. This variability suggests that PL present in DSPR preparations is adventitious. The tight binding of PL to reductase is demonstrated by our inability to remove the PL by gentle dialysis techniques. However, ultrafiltration, involving repeated concentration and dilution in a pressurized, stirred cell, results in partial removal of the bound PL.

Detergent-Solubilized Porcine Liver Reductase with and without Manganese. Figure 2 presents ^{31}P NMR spectra of DSPR with (A) and without (B) added manganese ions. The extent of Mn(II)-induced paramagnetic broadening of the ^{31}P resonances of steapsin-solubilized reductase has previously been used to provide a qualitative indication of the degree of solvent exposure of the different cofactors bound to the enzyme. Addition of manganese resulted in this case, as expected, in the complete disappearance of the free P_i signal appearing at 3.0 ppm, as well as the enhancement of the signal-to-noise ratios of the signals at 4.0 ppm attributable to bound FMN

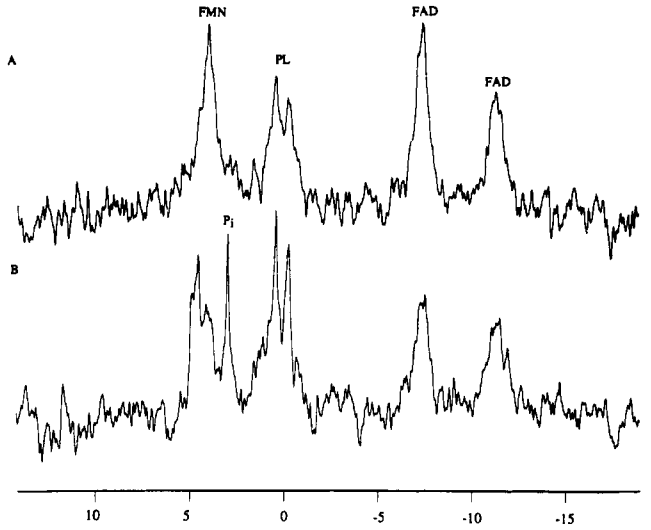

FIGURE 2: ^{31}P NMR spectra of NADPH–cytochrome P450 reductase from porcine liver solubilized with Emulgen 913 at pH 7.7 at 10 °C, with (A) and without (B) added Mn(II) ion: (A) 0.20 mM enzyme in the presence of 0.25 mM $MnSO_4$, 75 000 transients, line broadening 10 Hz; (B) same conditions as in (A) without $MnSO_4$, 120 000 transients, line broadening 10 Hz.

and at −7.3 and −11.3 ppm (Figure 2A) assigned to bound FAD. The same behavior was seen previously with the steapsin-solubilized reductase preparation in the presence of added manganese, which was attributed to a decrease in the spin–lattice relaxation times of these signals (Otvos et al., 1986). In the detergent-solubilized reductase preparation, shown in Figure 2, there is an extra signal at 4.5 ppm which is slightly downfield of the normal resonance of the bound FMN (4.0 ppm). In the presence of manganese (Figure 2A), this extra resonance is no longer detectable. Since a resonance at this position was not seen in any other DSPR preparation, we tentatively attribute it to an unknown phosphate monoester impurity. Finally, the putative PL signals have become much broader in the presence of manganese, suggesting partial exposure of the PL groups to bulk solvent.

Tissue-Purified, Detergent-Solubilized Rat Liver Reductase with and without Manganese. Figure 3 compares the spectra of the detergent-solubilized tissue-purified rat liver reductase (DSRR), with (A) and without (B) added $MnSO_4$. As in the cases of steapsin- and detergent-solubilized porcine reductases, the addition of manganese ions to DSRR resulted, as expected, in the complete broadening of the P_i resonance at 3.0 ppm (Figure 3A). In addition, there was a simultaneous increase in the signal-to-noise ratios of the bound flavin resonances despite the fact that the spectrum was acquired with fewer transients. The resonance(s) attributable to residual PL at ~0.0 ppm was (were) also significantly broadened upon the addition of manganese.

Cloned, Detergent-Solubilized Rat Liver Reductase with and without Manganese. Figure 4 contains the ^{31}P NMR spectra of the wild-type reductase, purified from *E. coli* harboring a plasmid containing the rat liver reductase gene (Shen et al., 1989), with (A) and without (B) added Mn(II) ions. It is clear that the inorganic phosphate signal at 3.1 ppm, as well as the resonance at 0.5 ppm due to PL, is completely broadened when manganese is added, while the 2′-AMP signal (2.0 ppm) is only minimally affected. The spectrum also exhibits a set of two new resonances, at −5.5 and −10.5 ppm (Figure 4B), just downfield of the "normal" FAD resonances. The relative amplitudes of these signals, compared to the FAD resonances, are variable from preparation to preparation. Both the cloned (Figure 4B) and rat liver (Figure 3B) reductases

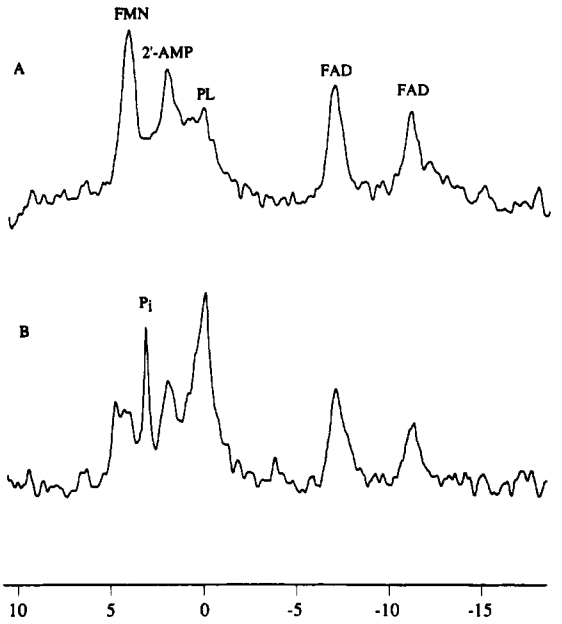

FIGURE 3: ^{31}P NMR spectra of NADPH–cytochrome P450 reductase from rat liver solubilized with Renex-690 at pH 7.7 at 10 °C, with (A) and without (B) added Mn(II) ion: (A) 0.15 mM enzyme in the presence of 0.20 mM $MnSO_4$, 90 000 transients, line broadening 20 Hz; (B) same as in (A), except for the absence of Mn(II), 163 000 transients.

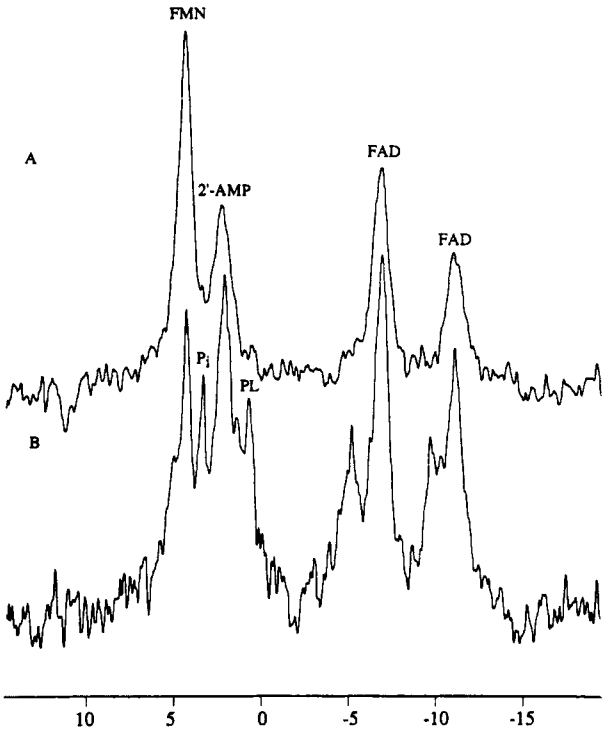

FIGURE 4: ^{31}P NMR spectra of cloned rat liver (wild-type) NADPH–cytochrome P450 reductase solubilized with Triton X-100 at pH 7.7 at 10 °C, with (A) and without (B) added Mn(II) ion: (A) 0.29 mM enzyme with added Mn(II) (0.40 mM), 117 000 transients, line broadening 15 Hz; (B) same conditions as in (A) without added manganese, 139 000 transients.

exhibit "PL" resonances near 0 ppm, although their line widths and actual chemical shifts are slightly different.

Cloned, Detergent-Solubilized Rat Liver Reductase (Asp_{140} Mutant) with and without Manganese. The mutation at Tyr_{140} to Asp_{140} was found to perturb the enzyme activity (the mutant exhibits only 20% of the wild-type activity for cytochrome *c* reduction). This reduction in activity, combined with the

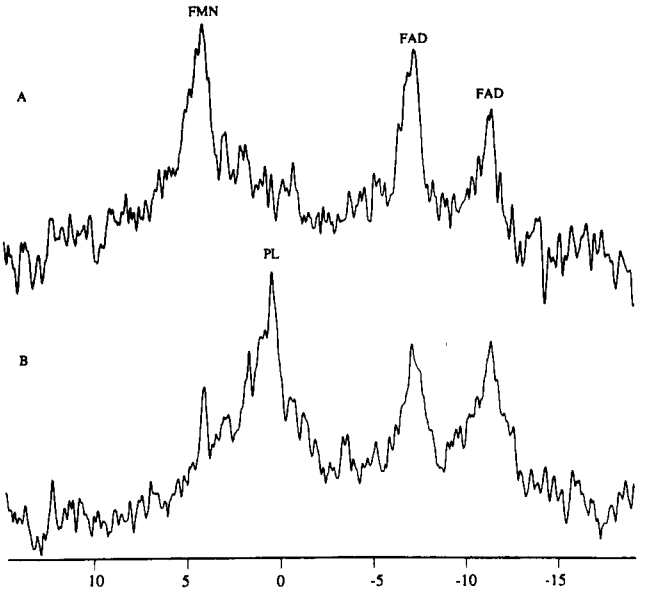

FIGURE 5: ^{31}P NMR spectra of cloned rat liver mutant (Tyr_{140} to Asp_{140}) NADPH–cytochrome P450 reductase solubilized with Triton X-100 at pH 7.7 at 10 °C, with (A) and without (B) added Mn(II) ion: (A) 0.20 mM enzyme plus 0.26 mM $MnSO_4$, 125 000 transients, line broadening 15 Hz; (B) same as in (A) without Mn(II), 204 000 transients.

observation that the Asp_{140} mutant contains both FAD and FMN (Shen et al., 1989), provided a strong incentive to scrutinize this mutant preparation by ^{31}P NMR spectroscopy. In Figure 5 are shown the ^{31}P NMR spectra of the Tyr_{140} to Asp_{140} mutant enzyme in the presence (A) and absence (B) of manganese. The chemical shifts of the flavins are the same as those of the cloned reductase, within experimental error. The NMR signal from FMN at 4.0 ppm (Figure 5B) is much smaller than the FAD signals, but the FMN signal recovers to full intensity when manganese is added to the sample (Figure 5A). One notable deviation in the Asp_{140} mutant is the failure to see the resonances at −5.5 and −10.5 ppm that were seen in spectra of cloned, wild-type reductase preparations. A very strong signal can also be observed at a chemical shift of 0.4 ppm, presumably arising from tightly bound phospholipid.

Cloned, Detergent-Solubilized Rat Liver Reductase (Asp_{178} Mutant) with and without Manganese. The Tyr_{178} to Asp_{178} mutation, on the other hand, does not bind FMN and contains only approximately half of the FAD of its native counterpart (Shen et al., 1989). Figure 6 displays the ^{31}P NMR spectra of a manganese addition experiment on the Asp_{178} mutant. The resonances at 1.7 ppm, due to 2′-AMP (Figure 6A), and at 3.1 ppm, due to P_i (Figure 6B), are present in addition to a strong PL signal resonating at about 0.5 ppm (Figure 6B). In agreement with the flavin content determination, the ^{31}P spectra of the Asp_{178} mutant exhibits no discernible FMN signal at 4.0 ppm and the FAD resonances are extremely weak. The addition of manganese broadens the P_i as well as the PL signal beyond detection (Figure 6A).

Phospholipid Analysis. Selected NMR samples were analyzed further to determine the nature of any PL present. (The data are not shown for any of the phospholipid analyses.) The extracted PLs were identified by comparison of their R_f values on TLC with those of authentic samples (see Materials and Methods). In experiments with tissue-purified porcine liver preparations, extractable PL was determined in all of the samples that contained NMR-detectable PL, whereas those preparations without NMR-detectable PL tested negative in PL analyses. In tissue-purified rat liver enzyme preparations,

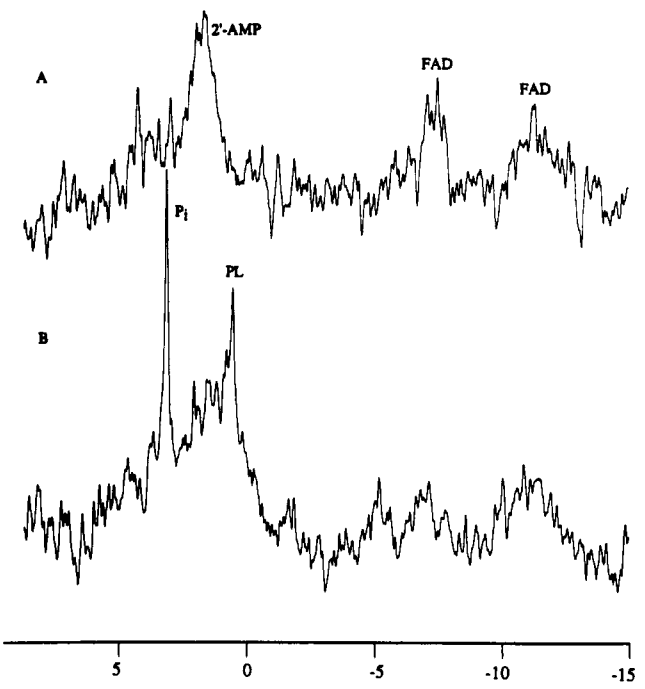

FIGURE 6: ^{31}P NMR spectra of cloned rat liver mutant (Tyr_{178} to Asp_{178}) NADPH–cytochrome P450 reductase solubilized with Triton X-100 at pH 7.7 at 10 °C, with (A) and without (B) added Mn(II) ion: (A) 0.18 mM enzyme plus 0.24 mM $MnSO_4$, 167 000 transients, line broadening 15 Hz; (B) same as in (A) without Mn(II), 92 000 transients.

which exhibited no PL in the ^{31}P NMR spectra, no evidence of extractable PL was found. Due to the lack of sufficient enzyme, the preparations that showed the presence of PL in TLC analysis could not be probed by ^{31}P NMR. The particular rat liver preparation that showed a strong PL signal (Figure 3) was not analyzed for its PL content. In addition, the PL extracts of the reductase from rat liver preparations exhibited large quantities of what appears to be detergent carried over during enzyme purification. The cloned-expressed enzymes (wild type, as well the mutants Asp_{140} and Asp_{178}), on the contrary, showed very little detergent (Triton X-100, $R_f = 0.80$) but displayed significantly larger quantities of the phospholipids, PE ($R_f = 0.63$) and PC ($R_f = 0.33$), evident from comparison with authentic standards on the TLC plate. In addition, free fatty acid ($R_f = 0.75$) in very small amounts was also observed. A few of the samples were further analyzed by isolating the PL bands from the TLC plate followed by quantitation of phosphorus. It appears that the *E. coli*-expressed reductase contains both PE and PC, with the PE being the predominant species, while the nature of the PL present in the pig liver reductases was not determined.

Discussion

In spite of the extensive library of information available on NADPH–cytochrome P450 reductase, several aspects of the enzyme are still only poorly understood. Although the sequence of electron flow is known, i.e., NADPH to FAD to FMN to heme protein, the complex series of electron-transfer reactions catalyzed within the reductase flavoprotein between FAD and FMN are still not understood at the molecular level. Another deficiency in our knowledge of this enzyme concerns the environments of the flavin prosthetic groups which could influence or participate in electron transfer between them. A first step would be to determine the amino acid residues involved in binding the flavin ligands to the reductase molecule. With the determination of the sequence homology with other

flavin-binding proteins (Porter & Kasper, 1986; Porter, 1991), this goal is closer to achievement. Our laboratories have determined the locus of the radical of the air-stable semiquinone to be the FMN moiety utilizing ^{31}P NMR (Otvos et al., 1986). This work utilized the steapsin-solubilized porcine liver reductase preparation exclusively. In the present work, we have extended our studies to include the comparative examination of NADPH–cytochrome P450 reductases purified from different tissues and sources by detergent solubilization, including cloned, *E. coli*-expressed forms of rat liver reductase, utilizing ^{31}P NMR as the main diagnostic tool. These experiments have permitted a deeper insight into their structure–function relationships.

In general, the ^{31}P spectra of the steapsin- and detergent-solubilized reductases from pig liver were found to be very similar (Figure 1). By comparison with the published values, the detergent reductase resonances can be assigned without difficulty to the phosphate groups of bound FMN, free inorganic phosphate (P_i), bound 2′-AMP, and bound FAD, in order of upfield chemical shifts. The chemical shifts of a given chemical species in reductase preparations purified from different species are normally found to be within a 0.1 ppm range.

The signals present in the 0 ppm range of the ^{31}P NMR spectra of various detergent-solubilized reductase preparations were tentatively attributed to bound phospholipid(s) (Masters et al., 1990; Narayanasami et al., 1991) by comparison with reported chemical shifts of phospholipid resonances, free and protein-bound (Henderson et al., 1974; Sotirhos et al., 1986). The appearance of a new signal at 0.0 ppm when phosphatidylinositol was added to a detergent-solubilized preparation also served to confirm our PL signal assignment (data not shown). It should be noted that this signal had been previously assigned to "covalently bound phosphorus" by another group (Bonants et al., 1985). However, in a recent publication Bonants et al. (1990) have concurred with our assignment of the ~0 ppm signal(s) to PL. The enzyme apparently binds phospholipid very tightly, as evidenced by the requirement for ultrafiltration, with exhaustive concentration and dilution, to diminish the PL signal.

The intensities as well as the chemical shifts of the putative PL signals are variable from preparation to preparation; these parameters also vary with the source of the enzyme, even though, in general, the phospholipids appear more regularly and in larger quantities in the NMR spectra of cloned rat liver reductase preparations, compared to the reductase from the mammalian sources used. This variability might mean that PL bound to the reductase is adventitious and is a consequence of the lipid environment in the particular cell type without any functional significance. This conclusion is supported by the lack of any meaningful correlation between the apparent PL content (estimated from NMR signal intensities relative to the FAD and FMN signals) and the specific activity.

The slight differences in the PL chemical shifts of different preparations may be attributable to different phospholipid species. This explanation is reasonable if one considers species differences. However, it can also be argued that the observed differences in the PL region of the ^{31}P spectra are the result of chemically identical phospholipids residing in different environments and that this variability in the microenvironment can be due either to variations in PL binding or to the different detergents used for solubilization of reductase from various species.

Several of the reductase preparations, purified from several different sources, both mammalian and expressed, were subjected to the extraction procedure of Bligh and Dyer (1959) to isolate the bound phospholipid in free form. The purified PLs were identified by thin-layer chromatographic (TLC) techniques. It was clearly demonstrated that those preparations displaying the "PL" signal in ^{31}P NMR spectra also showed PL bands on TLC plates, and vice versa. The results indicate that at least in the *E. coli*-expressed forms of reductase the major phospholipid in phosphatidylethanolamine (PE), with phosphatidylcholine present in lesser amounts. Given that PE is the most prevalent form of PL in *E. coli* (Randle et al., 1969), this conclusion is tenable.

The ^{31}P signals of bound FMN and FAD in detergent-solubilized reductase from different sources exhibit, in general, much larger line widths than those from steapsin-solubilized preparations (70–100 Hz versus about 40 Hz). For such tightly-bound cofactors, it is expected that their line widths would be directly related to the rotational correlation time of the enzyme, which in turn is proportional to its molecular weight. The presence of the extra N-terminal segment, which is absent in the steapsin-solubilized enzyme, may be responsible for the greater line widths in the case of the detergent-solubilized enzyme, simply by virtue of increasing the protein size. However, another explanation that better accounts for the larger magnitude of the line width increases is that the hydrophobic nature of the N-terminal segment induces protein aggregation. Preliminary data from sedimentation velocity experiments, performed with *E. coli*-expressed reductase at about 20% of the normal NMR concentrations, support this hypothesis. The hydrophobic, detergent-solubilized enzyme, in our experiments, exhibits a distribution of $s_{20,w}$ values ranging between 10 and 25 S, with the majority of the enzyme possessing a sedimentation coefficient of 18 S. The hydrophilic form of the enzyme, in which the hydrophobic N-terminus has been cleaved with trypsin, on the other hand, seems to contain a single species with an $s_{20,w}$ value of 4.2 S (J. C. Hansen, R. Narayanasami, and B. S. S. Masters, unpublished observations). These results suggest that, at least under the conditions employed in the sedimentation velocity experiments, the *E. coli*-expressed enzyme has a propensity to aggregate. We can safely extend this conclusion to include the detergent-solubilized mammalian reductase forms, all of which have the hydrophobic N-terminus. Previously, French et al. (1980) have shown that rabbit liver reductase exists as a hexamer (apparent molecular weight = 450000) by gel exclusion chromatography.

The net result of this aggregation phenomenon would be to shorten the T_2 relaxation times of the phosphorus nuclei of the flavin cofactors, thereby producing broader signals. The increased correlation time of the aggregated reductase would also be expected to lengthen T_1 relaxation times significantly, which are already relatively long (ca. 3–6 s) for the presumably unaggregated steapsin-solubilized enzyme (Otvos et al., 1986). The combination of broader resonances and longer T_1 values make NMR studies of the detergent-solubilized reductase much more difficult to perform, necessitating long acquisition times of up to 45 h required to overcome the poor signal-to-noise ratios.

For observing the ^{31}P signals of relatively solvent-inaccessible cofactors such as FMN and FAD, the addition of the paramagnetic relaxation agent, Mn(II), was found to provide a partial solution to the problem of low detection sensitivity. As seen in Figures 2–6, the signal-to-noise ratios for the FMN and FAD resonances of samples containing added Mn(II) were found in all cases to be greater than that for the corresponding samples without Mn(II), even though the former spectra were all acquired with fewer transients. This effect, also observed

previously for the steapsin-solubilized enzyme, is attributed to a reduction in the long T_1 values of the FMN and FAD signals, which causes each resonance to be less attenuated by the effects of incomplete relaxation between pulses (Otvos et al., 1986). Although the paramagnetic contribution of Mn(II) to overall T_1 relaxation rate of these resonances is relatively large (because T_1 relaxation is normally inefficient), the same is not true for T_2 relaxation. Because the natural T_2 relaxation rates are already so large, owing to the long correlation time of the enzyme, Mn(II) exerts little or no additional influence on the already broad line widths.

The same behavior was not observed for the phospholipid (PL) signals, which were found to be broadened significantly by Mn(II) in preparations of tissue-purified reductase (Figures 2 and 3) or broadened beyond detection in cloned–expressed reductase samples (Figures 4–6). These observations indicate that the phosphate-containing portions of the bound PL are much more accessible to bulk solvent than bound FMN or FAD.

Similarly, the "extra" pair of upfield resonances appearing at −5.5 and −10.0 ppm in spectra of cloned rat liver reductase (Figure 4B) apparently originate from a phosphodiester-containing species that is bound to the reductase in a manner that exposes this group to bulk solvent, since the resonances broaden beyond detection when Mn(II) is added (Figure 4A). These two signals are tentatively assigned to FAD bound in a different orientation or at a different site in the enzyme molecule. A similar explanation was proposed to account for the two ^{31}P signals observed for FMN in the case of steapsin-solubilized reductase (Otvos et al., 1986). The intensities of these two pairs of resonances are not consistent from preparation to preparation. Although the −10.0 ppm resonance may arise from free FAD, the line width is too broad to be consistent with this explanation. Moreover, the relative intensities of the signals at −5.5 and −10.0 ppm are very similar in all the preparations. Thus, if the −10.0 ppm resonance is attributed to free FAD, the origin of the −5.5 ppm resonance cannot be satisfactorily explained. In addition, free FAD would be expected to be removed from the enzyme solution during the ultrafiltration performed routinely prior to NMR experiments. The possibility that free FAD is formed during the NMR experiment can also be discounted since this would be reflected in reduced specific activity of the enzyme. No unusually large loss of activity, other than the usual loss due to storage and handling, was noted.

All of these observations point to the possibility that the two resonances in question arise from a population of FAD that is bound to the reductase in a different fashion or at a different site than those FAD molecules giving rise to the well-established −7.3 and −11.3 ppm signals (Otvos et al., 1986). The large differences between the chemical shifts of the resonances of the two different FAD moieties (−5.5 and −7.3 ppm versus −10.0 and −11.3 ppm) point to the fact that the chemical environments must be significantly different for these two sets of FAD molecules. The fact that this unusual set of resonances appears only in the case of the cloned reductase suggests a role for the host organism (*E. coli*) in determining the microenvironment of the flavins.

Furthermore, as already mentioned, the addition of manganese ions to the cloned reductase preparation dramatically broadened the two resonances at −5.5 and −10.0 ppm, while (as in the case of the purified reductase) the line widths of the "normally bound FAD" signals were unaffected (Figure 4). This provides additional evidence for the suggestion that the FAD has multiple microenvironments in the enzyme. The paramagnetic broadening experiments also indicate that the pyrophosphate portion of the normally bound FAD ligands are more deeply buried in the protein and do not experience significant interaction with bulk solvent.

The inability of Mn(II) to induce line broadening of the flavin resonances in the ^{31}P NMR spectra of reductases from all different sources can be accounted for by one of two possibilities. It suggests either that the phosphate groups of the flavins are effectively shielded from bulk solvent or that any contribution to the T_2 relaxation process is negligibly small due to the already small T_2 values. Another aspect of the detergent-solubilized reductase preparations, as already mentioned, is that the phosphate groups of the phospholipid moieties experience significant interaction with the bulk solvent, indicated by the large paramagnetic line broadening of the ^{31}P NMR signals induced by added Mn(II).

Therefore, it is of great interest and importance to determine the effects of manipulating the amino acid residues involved in FMN binding on the NMR properties, such as line widths, signal intensities, and chemical shifts. Since these parameters would be sensitive to changes in binding environments of the cofactors, useful insight can be gained into the nature of the interaction between the various ligands and the reductase.

Accordingly, Shen et al. (1989) have expressed the rat liver reductase in *E. coli*. Through site-directed mutagenesis they were able to create several mutants of the cloned reductase at key amino acid residues involved in FMN-binding. In one such mutant, Tyr_{178} was converted to Asp_{178}. This mutant does not bind FMN and nor does it transfer electrons to cytochrome *c*, indicating that the Tyr_{178} residue is essential for the enzyme to interact with the aromatic isoalloxazine moiety of FMN. The $Tyr_{140} \rightarrow Asp_{140}$ mutant exhibits 20% of the wild-type activity and binds FMN. On the other hand, when either one or both of the two Tyr residues are replaced with Phe, the mutants have similar affinities for flavin-binding as the wild-type reductase. However, the electron-transfer properties of the mutants at position 140 are slightly different from those of the wild type (Shen et al., 1989).

The Asp_{140} mutant was examined by ^{31}P NMR to help define the role that Tyr_{140} plays in the binding interactions between the FMN cofactor and the protein molecule and also to see if the loss of the aromatic residue leads to any other structural perturbations. Referring to the NMR spectra in Figure 4, this enzyme did not exhibit any significant deviation from the wild-type enzyme. The only notable difference between the spectrum of the wild type and that of the Asp_{140} mutant is the absence of twin peaks at −5.5 and −10.0 ppm in the FAD region in the mutant. One interpretation for this observation is that changing the Tyr_{140} residue, thought to be involved in binding FMN, to Asp_{140} has somehow altered the FAD-binding domain, leading to only one stable microenvironment for bound FAD.

In conclusion, the ^{31}P NMR spectra of reductases purified from different species or solubilized using different detergents are very similar. The chemical shifts of the bound flavins, 2′-AMP, and phospholipids are indistinguishable, i.e., within 0.1 ppm, among the preparations. However, there are differences in the phospholipid resonances and in the appearance of additional FAD signals at −5.5 and −10.0 ppm in the cloned, wild-type preparations. ^{31}P NMR has proved to be an extremely valuable tool in studying structure–function relationships in NADPH–cytochrome P450 reductase, a unique mammalian flavoprotein dehydrogenase known to contain both FAD and FMN as functional, redox prosthetic groups. The merit of this approach has been confirmed recently by the

studies of Bonants et al. (1990), who have corroborated our previous results (Otvos et al., 1986) and now report that all phosphorus-containing resonances are accounted for by non-convalently bound prosthetic groups, inorganic phosphate, nucleotides, or phospholipid, as we have reported in preliminary results (Masters et al., 1990; Narayanasami et al., 1991).

Future directions will include the examination of various FMN- and FAD-free, as well as cloned–expressed, preparations of NADPH–cytochrome P450 reductase with labeled flavin analogues, using techniques such as those utilized with the phosphorothioate derivative of FMN (Calhoun et al., 1987), in ^{13}C and ^{15}N NMR experiments in order to determine differences in conformation conferred by specific modifications or mutations. These experimental approaches are adjunct solution structure techniques to the determination of high-resolution X-ray structure, toward which efforts are in progress in our laboratory.

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Albumins Activate Peptide Hydrolysis by the Bifunctional Enzyme LTA_4 Hydrolase/Aminopeptidase†

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ABSTRACT: Albumins from several species activated the bifunctional, Zn^{2+} metalloenzyme aminopeptidase/leukotriene A_4 hydrolase (EC 3.3.2.6). Bovine serum albumin, 1 mg/mL, increased hydrolysis of L-proline-*p*-nitroanilide and leucine-enkephalin by 12-fold and 7-fold, respectively. The apparent K_m for L-proline-*p*-nitroanilide was inversely proportional to the albumin concentration from 0 to 1 mg/mL, declining from 9.4 to 0.7 mM without an appreciable change in apparent V_{max}. These data imply a random activation process in which the enzyme–activator complex is catalytically dominant. Hill plots indicated a 1:1 stoichiometric relationship between albumin and enzyme. Secondary plots of slope versus the reciprocal of albumin concentration indicated that it binds to the enzyme with an affinity constant of 0.9 μM. The pH optimum of the nonactivated enzyme occurred at pH 8; the albumin-activated enzyme had an optimum near pH 7. Neither ultrafiltration nor dialysis of albumin altered its activating effect, but boiling abolished it. Albumin did not affect other cytosolic or microsomal leucine aminopeptidases, or γ-glutamyltransferase. Albumin functions as a nonessential activator, since enzymatic activity was always detectable in its absence. Chloride ions, which activate other Zn^{2+} metalloenzymes, also activated leukotriene A_4 hydrolase/aminopeptidase with an EC_{50} = 50 mM, increasing its initial velocity 2.2-fold in the absence of albumin. Zn^{2+} activated the enzyme, increasing its apparent V_{max} but not its apparent K_m, suggesting it replaced Zn^{2+} lost from the active site, especially at acidic pH. At concentrations greater than 30–50 μM, Zn^{2+} was inhibitory. Albumin mitigated the effect of chloride, but not the effect of Zn^{2+} or that of the competitive inhibitor, captopril. Several other proteins including gelatin, bovine or human γ-globulins, human α_2-macroglobulin, and bovine α-fetoprotein did not stimulate the aminopeptidase activity of LTA_4 hydrolase. Our results suggest that an interaction between LTA_4 hydrolase/aminopeptidase and albumin causes a conformational change in the enzyme, leading to an increased affinity for peptides.

Leukotriene (LT)[1] A_4 hydrolase (EC 3.3.2.6) is a bifunctional Zn^{2+} metalloenzyme (Malfroy et al., 1989; Vallee & Auld, 1990; Toh et al., 1990; Haeggstrom et al., 1990a) which catalyzes the formation of LTB_4 (Radmark et al., 1984; Evans et al., 1985; McGee & Fitzpatrick, 1985) and the hydrolysis of amide derivatives of *p*-nitroaniline or naphthylamine (Minami et al., 1990; Haeggstrom et al., 1990b; Orning et al., 1991a,b). The K_m values for its amide substrates are 50–100 times larger than the K_m for LTA_4. However, the V_{max} values for both aminopeptidase and LTA_4 hydrolase catalysis are comparable; consequently, the k_{cat}/K_m ratio for amide hydrolysis is <2% that for LTB_4 formation (Minami et al., 1990; Orning et al., 1991a,b). Kinetic data reported by different groups contain discrepancies which are relevant to any proposed physiological roles for the aminopeptidase activity. Using homogeneously purified leukocyte enzyme at pH 8.0, 37 °C, with L-leucine-*p*-nitroanilide as a substrate, one group has reported a specific activity of 130 nmol of *p*-nitroaniline/(min·mg) (Haeggstrom et al., 1990b), a value substantially lower than the 500 nmol/(min·mg) we and others obtained using recombinant human leukocyte enzyme and various amide substrates under similar conditions (Minami et al., 1990; Orning et al., 1991a,b). Initially, we attributed this difference to the enzyme preparations, rather than to a minor item, inclusion of bovine serum albumin, 1 mg/mL, in our enzyme assay buffer. Albumin augments formation of LTB_4, an effect ordinarily attributed to sequestration and protection of LTA_4 from spontaneous, nonenzymatic hydration (Fitzpatrick et al., 1982; Maycock et al., 1982). We routinely included BSA in the assay buffer for LTA_4 hydrolase activity and, for consistency, also in the assay for the aminopeptidase

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[1] Abbreviations: LT, leukotriene; BSA, bovine serum albumin; PG, prostaglandin; fMLF, formyl-methionyl-leucyl-phenylalanine.

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