Catalytic Properties of Purified Forms of Rabbit Liver Microsomal Cytochrome P-450 in Reconstituted Phospholipid Vesicles†

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ABSTRACT: The catalytic properties of three forms of rabbit liver microsomal cytochrome P-450, LM_2, LM_3, and LM_4, have been examined by using six different substrates, in reconstituted phospholipid vesicles prepared by the cholate gel filtration technique from either microsomal phospholipids or egg yolk phosphatidylcholine and in a nonmembranous system reconstituted with dilauroylphosphatidylcholine. It was found that the catalytic properties of especially P-450_{LM_3} were markedly dependent on the type of reconstituted system employed. Thus, the substrate specificity of P-450_{LM_3} changed markedly upon incorporation of the protein into the vesicles, and different types of kinetics were obtained in the membranous as compared to the nonmembranous system. Assay of P-450_{LM_3}-catalyzed 6β-hydroxylation of androstenedione using various molar ratios of P-450 to P-450 reductase indicated a rapid lateral diffusion of the proteins in the vesicles but a more rigid arrangement in the dilauroylphosphatidylcholine system. P-450_{LM_2}-catalyzed reactions proceeded at a much lower rate in the egg yolk phosphatidylcholine vesicles than in the dilauroylphosphatidylcholine system. However, if the vesicles were prepared from microsomal phospholipids, the situation was changed and higher catalytic activities with a somewhat altered substrate specificity compared to the dilauroylphosphatidylcholine system were obtained. This could indicate a specific requirement of P-450_{LM_2} for certain membranous phospholipids. Some changes in catalytic activities were associated with concomitant changes in NADPH oxidase activity, indicating differences in the efficiency of the electron-transport flow from NADPH to cytochrome P-450 in the different types of reconstituted systems, whereas in other cases the "effectiveness" in the hydroxylation reaction, i.e., the amount of NADPH oxidized compared to the amount of product formed, was increased upon incorporation of the proteins into vesicles. The differences in catalytic properties between the membranous and the nonmembranous reconstituted systems are discussed in terms of (1) protein cluster formation, (2) facilitated interactions between P-450 and P-450 reductase, (3) facilitated binding of hydrophobic substrates to P-450, and (4) direct action of membranous phospholipid on P-450 in the former but not in the latter system.

The liver microsomal hydroxylase system is characterized by its remarkably broad substrate specificity. Endogenous compounds such as fatty acids, prostaglandins, and steroids as well as a large variety of environmental chemicals, e.g., drugs, insecticides, and precarcinogens, can serve as substrates. At least 15 types of reactions, e.g., N-dealkylation, N-oxidation, dehalogenation, and epoxidation, are attributed to the action of the liver microsomal hydroxylase system [cf. Coon et al. (1976)]. Many of these reactions result in the formation of compounds that exert toxic or carcinogenic effects. It is known that this cytochrome P-450 dependent metabolic activation is a prerequisite for the carcinogenic or toxic action of a variety of compounds such as polyaromatic hydrocarbons [cf. Ts'O & Gelboin (1978)], organic solvents (Pohl, 1978; Schaumburg & Spence, 1978), nitrosamines (Guttenplan et al., 1976), or monomers used in manufacturing plastics (Van Duuren, 1975). It may thus be of importance to elucidate the factors determining the specificity of the various P-450-catalyzed reactions.

The resolution of multiple forms of liver microsomal cytochrome P-450 with different but overlapping specificities (Haugen et al., 1975) allows the assumption that the presence or absence of one specific form of cytochrome P-450 is of importance in determining the specificity of the P-450-catalyzed oxygenation reaction. Since the substrate specificity studies hitherto performed with purified P-450 proteins have been carried out in a nonmembranous reconstituted system containing small amounts of dilauroylphosphatidylcholine, it was considered of importance to examine whether the substrate specificity and the catalytic properties of various types of cytochrome P-450 were influenced upon integration of the proteins into a membrane structure. For this purpose, apparently homogeneous preparations of cytochromes P-450_{LM_2}, P-450_{LM_3}, and P-450_{LM_4}[1] have been prepared and introduced together with homogeneous preparations of NADPH–cytochrome P-450 reductase into a phospholipid double layer by the cholate gel filtration technique previously described (Ingelman-Sundberg & Glaumann, 1977). The catalytic properties of the resulting vesicular preparations have been examined by using six different substrates, and, as comparison, incubations have also been performed with liver microsomes and with a reconstituted system containing small amounts of dilauroylphosphatidylcholine. It is shown that the catalytic properties of especially P-450_{LM_3} are markedly dependent on whether the enzyme is embedded in a membrane structure or not.

Materials and Methods

Materials. 7-Ethoxycoumarin was purchased from Aldrich Chemical Co. [4-^{14}C]Androstenedione was purchased from New England Nuclear and purified by thin-layer chromatography before use. [G-^{3}H]Benzo[*a*]pyrene was obtained from the Radiochemical Centre, purified by high-pressure liquid chromatography prior to use, and stored in the dark at −20 °C under nitrogen. Aminopurine and ethylmorphine were from Karolinska Apoteket, Stockholm. Cyclohexane (P.A.) and acetanilide were obtained from Merck, and androstenedione was from the Upjohn Co. NADPH and benzo[*a*]pyrene were purchased from Sigma.

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[1] Abbreviations used: P-450_{LM}, liver microsomal cytochrome P-450; P-450_{LM_2}, P-450_{LM_3}, and P-450_{LM_4}, forms of P-450_{LM} designated according to their electrophoretic properties; $NaDodSO_4$, sodium dodecyl sulfate.

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Methods. Cytochrome P-450 and NADPH–cytochrome P-450 reductase were purifed from liver microsomes obtained from phenobarbital-treated rabbits as previously described (Ingelman-Sundberg et al., 1979; Ingelman-Sundberg & Glaumann, 1980). The purification methods are based on those described by Haugen & Coon (1976) and Yasukochi & Masters (1976), respectively. The specific contents of the preparations were the following: cytochrome P-450$_{LM_2}$, 12.5–16.5 nmol/mg; cytochrome P-450$_{LM_3}$, 11.5 nmol/mg; cytochrome P-450$_{LM_4}$, 11.4 nmol/mg; and NADPH–cytochrome P-450 reductase, 12.2 nmol of flavin/mg when flavin was quantitated by the absorption at 456 nm with the absorption coefficient 10.7 mM^{-1} cm^{-1} (Iyamagi & Mason, 1973). The preparations were homogeneous when analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis (Ingelman-Sundberg & Glaumann, 1980) in the Laemmli system (Laemmli, 1970). The specific contents of the cytochrome P-450 preparations indicating only 50–70% homogeneity are apparently explained by heme losses during the purification procedure. However, contaminating proteins in the preparations having identical molecular weights cannot be excluded.

Unilamellar phospholipid vesicles containing purified forms of cytochrome P-450 and NADPH–cytochrome P-450 reductase were prepared by the cholate gel filtration technique as previously described (Ingelman-Sundberg & Glaumann, 1977; Ingelman-Sundberg et al., 1979). Cytochrome P-450 was measured according to Omura & Sato (1964). NADPH–cytochrome P-450 reductase was measured as NADPH–cytochrome *c* reductase activity at 22 °C in 50 mM potassium phosphate buffer.[2] Protein was determined according to Lowry et al. (1951) with bovine serum albumin as standard. Hydroxylations of androstenedione were assayed according to Hrycay et al. (1976). O-Dealkylation of ethoxycoumarin was measured as described by Prough et al. (1978). N-Demethylation of aminopurine and ethylmorphine was recognized by the formation of formaldehyde according to the procedure of Nash (1953). Hydroxylation of benzo[*a*]pyrene was measured as the amount of radioactive metabolites eluting together with authentic 3-hydroxybenzo[*a*]pyrene in a reversed-phase high-pressure liquid chromatography system described elsewhere (Halpert et al., 1979). Liver microsomes were prepared from phenobarbital-treated rabbits as described by Strobel & Dignam (1978).

Results and Discussion

Hydroxylation of Androstenedione by Liver Microsomes. Androstenedione was hydroxylated in positions 6β, 7α, and 16 by liver microsomes from phenobarbital-treated rabbits. Substrate curves for these hydroxylation reactions are shown in Figure 1. 6β-Hydroxylation proceeded at a rate ten times higher than the other reactions, i.e., about 2 nmol of product min^{-1} $nmol^{-1}$ of microsomal P-450 was formed. The apparent K_m value of androstenedione for the 6β-hydroxylase(s) was much higher (0.2 mM) than for the other hydroxylases. Furthermore, the kinetics for the 6β-hydroxylation reaction were not of Michaelis–Menten type. A sigmoidal substrate curve and a Lineweaver–Burk plot concave upward (not shown in figure) characterized the kinetics obtained.

Hydroxylation of Androstenedione by Reconstituted Systems. Androstenedione metabolism was examined in reconstituted phosphatidylcholine vesicles containing NADPH–cytochrome P-450 reductase and cytochromes P-450$_{LM_2}$, P-450$_{LM_3}$, or P-450$_{LM_4}$ and in preparations where the proteins, in identical amounts and proportions, had been incubated with 50 μg of dilauroylphosphatidylcholine for 5 min at 37 °C.

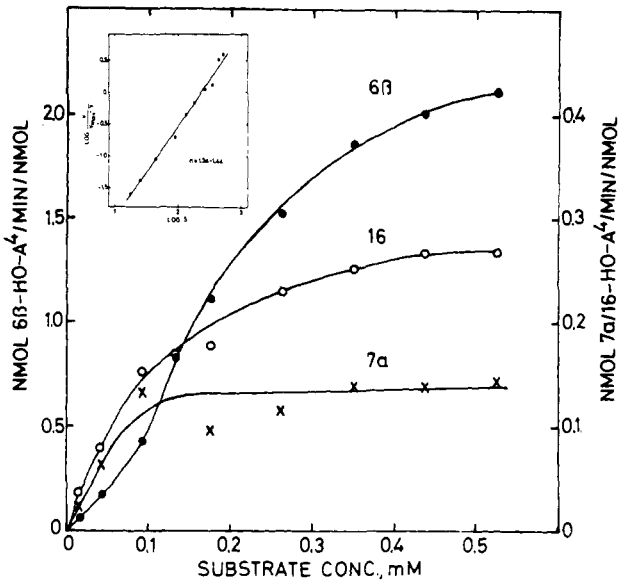

FIGURE 1: Hydroxylation of androstenedione in liver microsomes from phenobarbital-treated rabbits as a function of substrate concentration. Incubations were performed at 37 °C for 10 min by using microsomes corresponding to 1 mg of protein in a total volume of 2 mL of 50 mM potassium phosphate buffer, pH 7.4. The substrate was added in 50 μL of acetone, and preincubation was performed for 5 min at 37 °C before the incubations were started by the addition of 0.5 mg of NADPH in 100 μL of water. The incubations were terminated by the addition of 10 mL of chloroform/methanol (2:1 v/v) and analyzed as described elsewhere. Control incubations were performed with boiled microsomal preparations. Insert: Hill plot of the 6β-hydroxylase activity.

It was found that in both types of reconstituted systems, P-450$_{LM_2}$ catalyzed 16-hydroxylation and P-450$_{LM_3}$ catalyzed 6β-hydroxylation of androstenedione while P-450$_{LM_4}$ did not hydroxylate the steroid in either system. Time curves for the two hydroxylations are shown in Figure 2. The initial rate of P-450$_{LM_3}$-catalyzed 6β-hydroxylation was about ten times higher when phosphatidylcholine vesicles were used as the reconstituted system instead of the nonmembranous dilauroylphosphatidylcholine system. By contrast, 16-hydroxylation, catalyzed by P-450$_{LM_2}$, proceeded at a rate much higher in the nonmembranous than in the membranous system (cf. Figure 2B).

Substrate curves for 6β-hydroxylation of androstenedione catalyzed by P-450$_{LM_3}$ in reconstituted egg yolk phosphatidylcholine vesicles and in the dilauroylphosphatidylcholine system are shown in Figure 3. Sigmoidal kinetics were obtained in the vesicular but not in the nonmembranous system. A Hill plot of the results obtained in the vesicular system yielded a straight line, and a Hill coefficient of 1.3–1.4 and a K_m value of 0.2 mM could be calculated (Figure 3). A Hill plot of the values obtained for 6β-hydroxylation by intact liver microsomes also gave a Hill coefficient of 1.3–1.4 (Figure 1) and a K_m value of 0.2 mM. Obviously, the kinetics for 6β-hydroxylation are identical in both types of membrane-bound systems but different in the dilauroylphosphatidylcholine system.

After examination of the rate of P-450$_{LM_2}$-catalyzed 16-hydroxylation at different substrate concentrations in the nonmembranous dilauroylphosphatidylcholine system, an apparent V_{max} for the reaction of 0.3 nmol of product $nmol^{-1}$ of

[2] The amount of NADPH–cytochrome P-450 reductase was calculated by using a specific activity of 63 000 units/mg of protein when measured at 30 °C in 300 mM phosphate buffer, pH 7.4. One unit is defined as the amount of enzyme that reduces 1 nmol of cytochrome *c* per min.

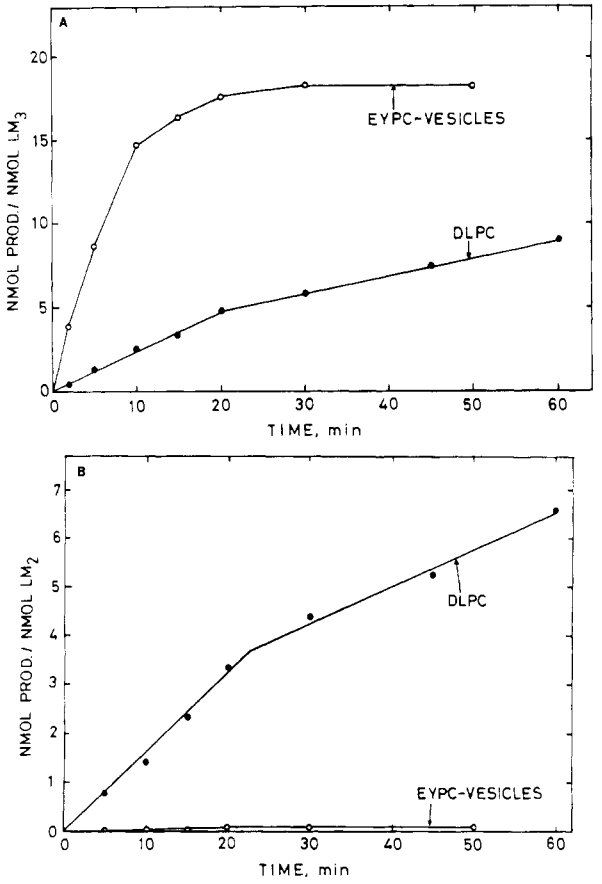

FIGURE 2: Time curves for P-450$_{LM_3}$-catalyzed 6β-hydroxylation (A) and P-450$_{LM_2}$-catalyzed 16-hydroxylation (B) of androstenedione in reconstituted phosphatidylcholine vesicles and in a system reconstituted with dilauroylphosphatidylcholine. Vesicles were prepared from 10 mg of egg yolk phosphatidylcholine, 5 nmol of P-450, and 2500 U of NADPH-cytochrome P-450 reductase by the cholate gel filtration technique. The vesicular incubations contained 0.5 nmol of P-450 and 200 U of P-450 reductase in a volume adjusted to 2 mL with 50 mM phosphate buffer, pH 7.4. Reconstitution with dilauroylphosphatidylcholine was performed by mixing 0.5 nmol of P-450, 200 U of P-450 reductase, and 50 μg of dilauroylphosphatidylcholine in 2 mL of phosphate buffer with subsequent incubation for 5 min at 37 °C. Incubations with substrate (350 μM) were performed as described in the legend to Figure 1.

P-450 min^{-1} and an apparent K_m of 0.3 mM were calculated. Michaelis–Menten kinetics were obtained in this case (not shown in the figure).

P-450$_{LM_3}$-Catalyzed 6β-Hydroxylation of Androstenedione. A finding of a Hill coefficient greater than 1 for a reaction can indicate positive cooperativity in the action of the enzyme molecules [cf. Koshland (1970) and Massa et al. (1975)]. One plausible explanation for the phenomenon can be that the P-450 molecules form clusters in the membrane (Peterson et al., 1976), thereby making cooperative action possible.

Reconstituted vesicles containing P-450$_{LM_3}$ to P-450 reductase in molar ratios of 13 or 1.0 were therefore prepared and incubated with increasing amounts of androstenedione. The results obtained are shown in Figure 4. The sigmoidal shape of the substrate curve was not abolished by decreasing the amount of P-450$_{LM_3}$ in the membrane. However, a smaller Hill coefficient (1.2) and a proportionally higher rate of 6β-hydroxylation at lower substrate concentration were obtained in the vesicles with a P-450$_{LM_3}$ to P-450 reductase molar ratio of 1.0.

When the enhancement of NADPH oxidase activity, mediated by increasing amounts of androstenedione in the vesicular preparations having a P-450$_{LM_3}$ to P-450 reductase

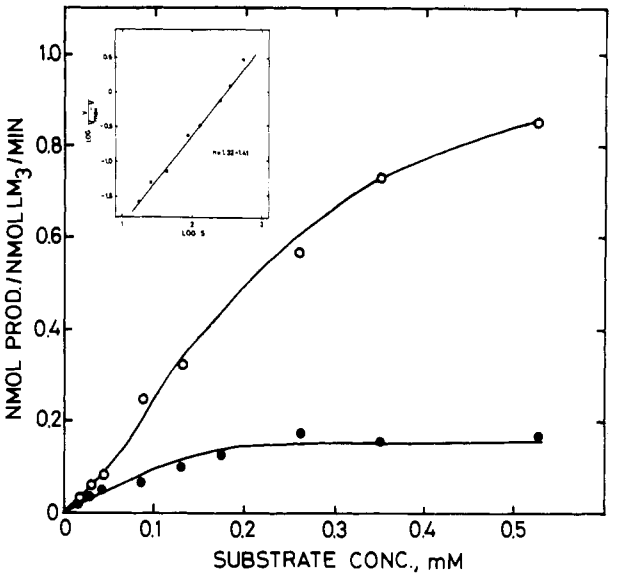

FIGURE 3: P-450$_{LM_3}$-catalyzed 6β-hydroxylation of androstenedione in reconstituted egg yolk phosphatidylcholine vesicles (○) and in the dilauroylphosphatidylcholine system (●) as a function of substrate concentration. Incubation conditions were as described in the legend to Figure 2. Insert: Hill plot of the 6β-hydroxylase activity in phosphatidylcholine vesicles.

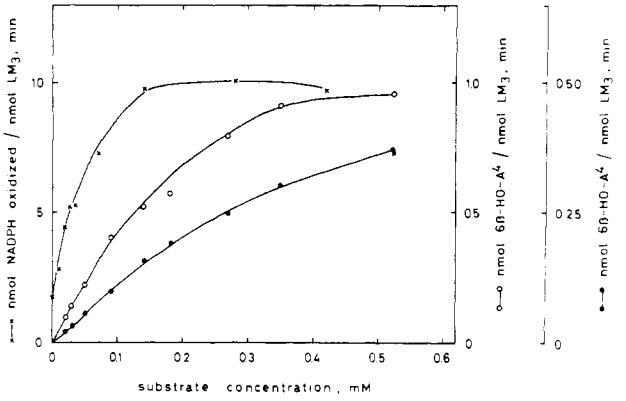

FIGURE 4: Androstenedione-induced NADPH oxidation in egg yolk phosphatidylcholine vesicles containing P-450$_{LM_3}$ (x) and P-450$_{LM_3}$-catalyzed 6β-hydroxylation of androstenedione in reconstituted vesicles containing a P-450$_{LM_3}$ to NADPH–cytochrome P-450 reductase molar ratio of 13 (●) and 1.0 (○), respectively. NADPH oxidation was measured by the decrease of absorption of NADPH at 340 nm and 37 °C by using vesicles in a volume adjusted to 1 mL with 50 mM potassium phosphate buffer, pH 7.4, corresponding to 0.15 nmol of P-450$_{LM_3}$ having a P-450 to P-450 reductase molar ratio of 13. 6β-Hydroxylation was assayed as described in the legend to Figure 2. Incubations were performed with vesicles corresponding to 0.47 and 0.13 nmol of P-450$_{LM_3}$, respectively.

molar ratio of 13, was measured spectrophotometrically, a different type of substrate curve was obtained (cf. Figure 4). A Lineweaver–Burk plot of these values yielded a straight line (Figure 5), indicative of Michaelis–Menten kinetics. The half-maximal effect was obtained at a substrate concentration of 24 μM. Evidently, the binding of androstenedione to P-450$_{LM_3}$, probably resulting in the increased electron transfer to the enzyme from NADPH, and the formation of the product, 6β-hydroxyandrostenedione, are two different processes; product formation is characterized by "cooperativity" and a high K_m value, NADPH oxidation by Michaelis–Menten kinetics and a half-maximal effect at low substrate concentrations.

If binding of the substrate to P-450 is correlated with increased NADPH oxidation as proposed by Sato and collab-

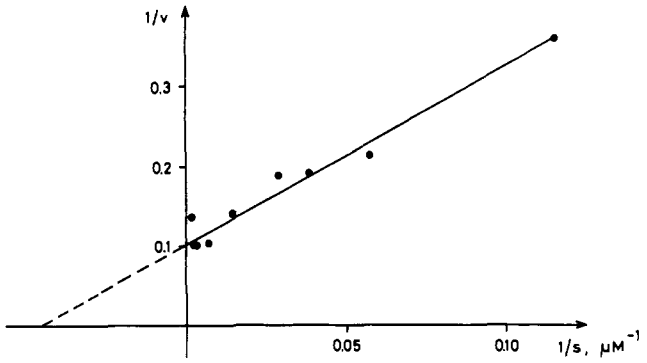

FIGURE 5: Lineweaver–Burk plot of androstenedione-induced NADPH oxidation in egg yolk phosphatidylcholine vesicles containing cytochrome P-450$_{LM_3}$, P-450 reductase, and phospholipid in a molar ratio of 13:1:1500. The values are taken from Figure 4.

orators (Imai et al., 1977), the "cooperativity" obtained could not be explained by slow diffusion of the substrate to P-450. The possibility was then considered that the "cooperativity" observed was due to H_2O_2 as a second activator.

H_2O_2 as a Possible Second Activator. Nordblom & Coon (1977) have shown that during P-450-catalyzed hydroxylation reactions in a reconstituted system, NADPH is utilized for two reactions: (1) hydroxylation of the substrate, NADPH + H^+ + O_2 + RH → $NADP^+$ + H_2O + ROH, and (2) oxidase activity, NADPH + H^+ + O_2 → $NADP^+$ + H_2O_2. Thus, the stoichiometry of the overall reaction reveals that the sum of the number of ROH and H_2O_2 molecules formed is equal to the number of NADPH molecules oxidized.

It is evident from Figure 4 that the production of hydrogen peroxide in the vesicular system probably is high compared to the amount of product formed. It has previously been shown that hydrogen peroxide efficiently can replace NADPH, P-450 reductase, and molecular oxygen in P-450-dependent hydroxylation reactions (Hrycay et al., 1975; Nordblom et al., 1976), and it was thus considered that the hydrogen peroxide formed in the system could activate P-450 molecules not reduced by NADPH and thus act as a second activator, thereby explaining the "cooperativity".

When the effect of increasing concentrations of hydrogen peroxide on the rate of P-450$_{LM_3}$-catalyzed 6β-hydroxylation of androstenedione in the reconstituted vesicles was examined, an apparent K_m of H_2O_2 for P-450$_{LM_3}$ of 9 mM was calculated, a value about 30 times lower than reported with P-450$_{LM_2}$ (Nordblom et al., 1976). Obviously, H_2O_2 efficiently activates P-450$_{LM_3}$. However, as evident from Figure 6, the "cooperativity" was not abolished when 50 mM H_2O_2 was used as the sole oxygen donor to P-450$_{LM_3}$ in the vesicles; a Hill coefficient of 1.31 was obtained. It may thus be concluded that these types of kinetics are not caused by the action of hydrogen peroxide as a second activator, and, furthermore, as pointed out above, they probably are not caused by slow diffusion of the substrate to the enzyme. Remaining possibilities include true cooperation between the P-450$_{LM_3}$ molecules when bound to the membrane or an androstenedione-mediated modulation of the membrane structure resulting in altered properties of P-450$_{LM_3}$ in membranes with high concentrations of the substrate. Since "cooperativity" is obtained in both microsomes and the egg yolk phosphatidylcholine vesicles, which have completely different compositions, the latter explanation seems less plausible.

Effect of the P-450$_{LM_3}$ to Reductase Ratio of Androstenedione-Dependent NADPH Oxidation and 6β-Hydroxylation. Much work has been concentrated on the rate-limiting step in the liver microsomal hydroxylase system [cf., e.g., Imai et

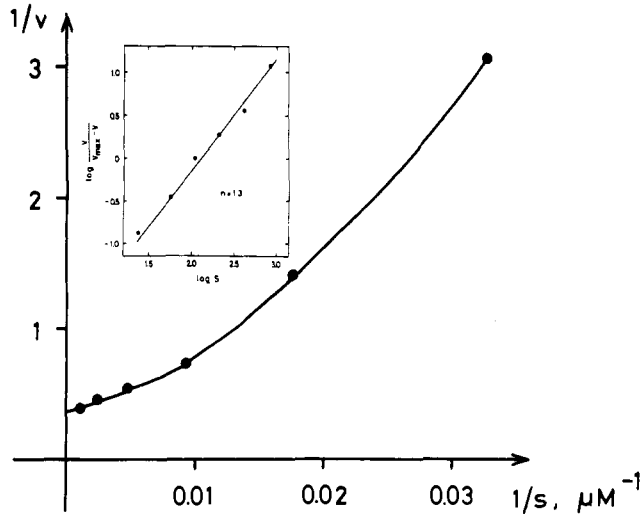

FIGURE 6: Lineweaver–Burk plot of hydrogen peroxide supported P-450$_{LM_3}$-catalyzed 6β-hydroxylation of androstenedione in reconstituted vesicles. The vesicles were prepared from 5 mg of egg yolk phosphatidylcholine and 5 nmol of P-450$_{LM_3}$ by using the cholate gel filtration technique. Incubations were performed with vesicles corresponding to 0.4 nmol of P-450 for 10 min at 37 °C with 50 mM H_2O_2 as the oxygen donor. Other conditions were as described in the legends to Figures 1 and 5. Insert: Hill plot of the 6β-hydroxylase activity.

al. (1977), Matsubana et al. (1976), and Miwa et al. (1978)]. From experiments with liver microsomes, it has been suggested that under some conditions and for certain substrates the amount of NADPH–cytochrome P-450 reductase in the membrane could be rate determining, but under other conditions the number of P-450 molecules will affect the overall hydroxylation rate (Miwa et al., 1978). Evidence has accumulated for the formation of a 1:1 catalytically functional complex between P-450 reductase and P-450$_{LM_4}$ by using the dilauroylphosphatidylcholine reconstituted system (Vatsis et al., 1979). However, since lateral diffusion of the protein components in the membrane occurs rapidly, it is not certain that 1:1 complexes will be formed between the reductase and P-450 in a fluid membrane and that the amount of P-450 reductase found here will be rate limiting. It was therefore considered of interest to study the effect of different ratios of P-450 to P-450 reductase on the NADPH oxidation and 6β-hydroxylation reactions induced by androstenedione.

In the experiments shown in Figure 7, the amount of the reductase was fixed, and the number of P-450$_{LM_3}$ molecules was increased to a P-450/P-450 reductase molar ratio of 22 in reconstituted phospholipid vesicles and in the dilauroylphosphatidylcholine system. The amount of lipid was varied in the different liposomal preparations so that the amount of protein was 9% of the total weight.

The results obtained (Figure 7) show that androstenedione-dependent NADPH oxidation and 6β-hydroxylase activities increase concomitantly as the P-450 to reductase ratio is increased in both the membrane-bound and the nonmembranous preparations. However, with the dilauroylphosphatidylcholine system, the maximal rate of NADPH oxidation and 6β-hydroxylation is obtained at a P-450 to reductase ratio of about 2 while in the membrane-bound system no saturation of the two activities was obtained even at a P-450 to reductase ratio of 22. These results indicate that in the nonmembranous system formation of 1:1 complexes between P-450 and the reductase takes place in agreement with the results obtained by Coon's group (Vatsis et al., 1979) and, furthermore, that the interdistribution of reductase molecules

Table I: Substrate Specificity of Cytochromes P-450$_{LM_{2-4}}$ in Reconstituted Phospholipid Vesicles Prepared from either Microsomal Phospholipids (MIC.P.L.) or Egg Yolk Phosphatidylcholine (EYPC) and in the Dilauroylphosphatidylcholine (DLPC) System [a]

	catalytic activity (nmol of product nmol^{-1} of P-450 min^{-1})						
	LM$_2$			LM$_3$		LM$_4$	
substrate	vesicles MIC.P.L.	vesicles EYPC	DLPC	vesicles EYPC	DLPC	vesicles EYPC	DLPC
androstenedione,[c] 350 μM	0.063	0.01	0.16	1.5	0.12	<0.001	<0.001
ethylmorphine, 1.3 mM	8.7	1.1	4.8	1.9	1.0	*b*	*b*
aminopurine, 2.3 mM	3.6	1.1	4.4	2.2	2.6	*b*	*b*
7-ethoxycoumarin, 50 μM	0.45	0.12	0.22	0.22	0.91	0.014	0.12
benzo[a]pyrene, 25 μM[d]	0.22	0.008	0.20	0.03	0.063	*b*	*b*
acetanilide, 1.3 mM	*b*	*b*	*b*	*b*	*b*	0.39	0.88

[a] Reconstitution was performed as described in the legend to Figure 2. Incubations in triplicate were performed at 37 °C for 10 min in a volume adjusted to 1 mL with 50 mM (100 mM when using LM$_4$) potassium phosphate buffer, pH 7.4. The incubations contained about 0.3 nmol of P-450 and 130 U of P-450 reductase. Equivalent conditions and protein preparations were used for the corresponding incubations with one type of cytochrome P-450. The different products were measured as described under Materials and Methods. [b] Not determined. [c] The products measured were 6β-hydroxyandrostenedione (LM$_3$) and 16-hydroxyandrostenedione (LM$_2$). [d] The product measured was 3-hydroxybenzo[a]pyrene.

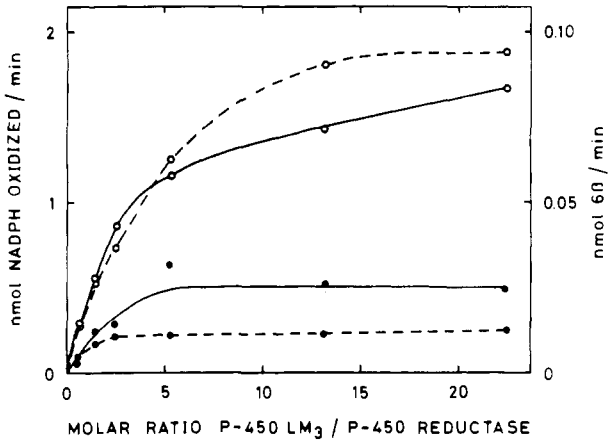

FIGURE 7: Androstenedione-induced NADPH oxidation and 6β-hydroxylation of androstenedione as a function of the molar ratio of NADPH–cytochrome P-450 reductase to cytochrome P-450$_{LM_3}$ in reconstituted phospholipid vesicles and in a system reconstituted with dilauroylphosphatidylcholine. Vesicles were prepared from egg yolk phosphatidylcholine, 2000 U of P-450 reductase, and varying amounts of P-450$_{LM_3}$ by the cholate gel filtration technique, giving vesicles having the molar ratios indicated of P-450$_{LM_3}$ to P-450 reductase. The amount of phospholipid was chosen to give a 9% (w/w) final concentration of protein. The dilauroylphosphatidylcholine system was reconstituted by mixing the same amounts of P-450 and P-450 reductase as obtained in the different vesicular preparations with 50 μg of dilauroylphosphatidylcholine in 1 mL of 50 mM potassium phosphate buffer, pH 7.4, followed by incubation for 5 min at 37 °C. NADPH oxidation was measured as described in the legend to Figure 4, and 6β-hydroxylation was measured as described in the legend to Figure 2. NADPH oxidation in vesicles (O--O); 6β-hydroxylation in vesicles (O—O); NADPH oxidation in the dilauroylphosphatidylcholine system (●--●); 6β-hydroxylation in the dilauroylphosphatidylcholine system (●—●).

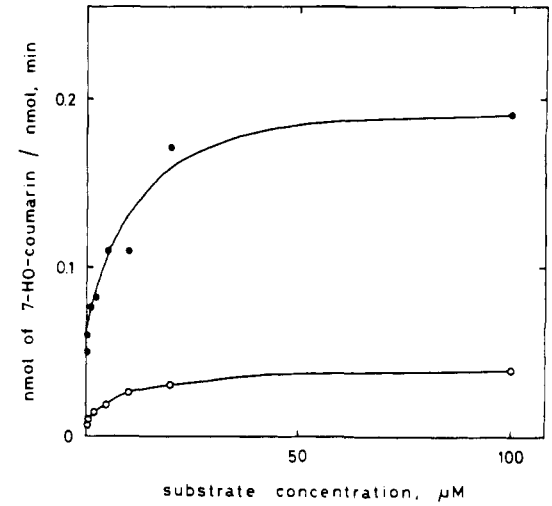

FIGURE 8: Cytochrome P-450$_{LM_3}$-catalyzed O-deethylation of 7-ethoxycoumarin in reconstituted egg yolk phosphatidylcholine vesicles (O) and in the dilauroylphosphatidylcholine system (●). The *O*-deethylase activity was determined spectrofluorometrically in 2 mL of 50 mM potassium phosphate buffer, pH 7.4. Incubations were performed with vesicles corresponding to 0.2 nmol of P-450 having a P-450/P-450 reductase molar ratio of 7.

between different P-450 molecules is slow. However, in the membranous system, a limited number of reductase molecules seems to be able to rapidly serve a great number of P-450 molecules, probably due to fast lateral diffusion in the membrane. If lateral diffusion of the hydroxylase system also occurs rapidly in intact microsomes, as is the case in the cytochrome b_5 system (Rogers & Strittmatter, 1974a,b), the molar ratio of P-450 to the reductase in intact liver microsomes of 20:1 (Estabrook et al., 1971) is more easily understood.

Kinetics of P-450$_{LM_3}$-Catalyzed O-Dealkylation of 7-Ethoxycoumarin. The kinetics of 7-ethoxycoumarin *O*-dealkylase were examined with membrane-bound and soluble preparations of P-450$_{LM_3}$. As evident from Figure 8, the reaction rate was only about 20% when the vesicular system was used compared to when the same amount of proteins was reconstituted with dilauroylphosphatidylcholine. Obviously, the situation here is reversed compared to when androstenedione was used as substrate (cf. above). Addition of saturating amounts of 7-ethoxycoumarin to the vesicles decreased the NADPH oxidation rate by 30% while after the same addition to the soluble system the NADPH oxidation was increased by 13% (cf. Table II). Nonlinear Lineweaver–Burk plots, indicative of two catalytic sites, are obtained in both cases. The apparent K_m values calculated, 0.25 μM and 5 μM, are not significantly different in either the membrane-bound or the soluble preparation. Thus, the decreased rate of O-dealkylation in the vesicles seems partly to be dependent on a diminished rate of electron transport from NADPH to P-450 in this system.

Substrate Specificity of Cytochromes P-450$_{LM_{2-4}}$ in Phospholipid Vesicles and in a Reconstituted System Containing Dilauroylphosphatidylcholine. Table I summarizes the hydroxylase activities of six different substrates catalyzed by cytochromes P-450$_{LM_{2-4}}$ in vesicles prepared from microsomal phospholipids or egg yolk phosphatidylcholine and in a nonmembranous reconstituted system containing dilauroylphosphatidylcholine. The vesicular incubations were performed with liposomes corresponding to 0.3 nmol of P-450 having a

Table II: Cytochrome P-450 Dependent NADPH Oxidation in Reconstituted Phospholipid Vesicles Prepared from either Microsomal Phospholipids (MIC.P.L.) or Egg Yolk Phosphatidylcholine (EYPC) and in the Dilauroylphosphatidylcholine System (DLPC)[a]

	NADPH oxidase activity (nmol of NADPH oxidized $nmol^{-1}$ of P-450 min^{-1})						
	LM_2			LM_3		LM_4	
substrate	vesicles MIC.P.L.	vesicles EYPC	DLPC	vesicles EYPC	DLPC	vesicles EYPC	DLPC
-	14.0	4.4	13.1	4.4	6.0	*b*	*b*
androstenedione, 350 μM	8.4	0.91	4.0	41	12	*b*	*b*
ethylmorphine, 1.3 mM	16.8	2.1	4.9	14	9.0	*b*	*b*
aminopurine, 2.3 mM	3.6	1.6	4.4	5.0	5.0	*b*	*b*
7-ethoxycoumarin, 50 μM	7.7	4.1	16.8	3.2	6.8	3.0	3.2
benzo[*a*]pyrene, 25 μM	*b*	6.8	13.0	6.4	3.8	*b*	*b*
cyclohexane, 2 mM	28.0	7.8	19.4	10.2	10.9	*b*	*b*
acetanilide, 1.3 mM	*b*	*b*	*b*	*b*	*b*	1.8	4.2

[a] NADPH oxidation was measured at 37 °C by following the decrease of absorption at 340 nm. The cuvette contained about 0.05 nmol of P-450 and 20 U of P-450 reductase from the preparations used in the experiments outlined in Table I in a total volume adjusted to 1 mL with 50 mM (100 mM when using LM_4) potassium phosphate buffer, pH 7.4. [b] Not determined.

P-450/P-450 reductase/phospholipid ratio of 3:1:2000. The nonvesicular incubations were performed with identical amounts and the same preparations of P-450 and P-450 reductase as used in the vesicular incubations but contained 50 μg of dilauroylphosphatidylcholine instead of membranous phospholipid. As seen in Table I, cytochrome $P\text{-}450_{LM_2}$ dependent hydroxylase activities generally proceeded at a rate 2.5–5 times higher in the nonmembranous dilauroylphosphatidylcholine system than in vesicles prepared from egg yolk phosphatidylcholine. When the NADPH oxidase activities were measured spectrophotometrically in the phosphatidylcholine vesicles and the dilauroylphosphatidylcholine preparations in the presence of the different substrates, it became evident that the diminished hydroxylase activity in the vesicles was correlated to a decreased rate of $P\text{-}450_{LM_2}$-dependent NADPH oxidase activity (Table II). However, if the vesicles were prepared from total microsomal phospholipids, both the $P\text{-}450_{LM_2}$-dependent hydroxylase activities and the NADPH oxidase activities were increased to levels higher than those obtained in the dilauroylphosphatidylcholine system (Tables I and II). Therefore, it seems likely that phospholipids other than phosphatidylcholine are of importance for reconstitution of $P\text{-}450_{LM_2}$-dependent activities in vesicles.

The different properties of $P\text{-}450_{LM_2}$-containing vesicles prepared either from microsomal phospholipids or from egg yolk phosphatidylcholine are illustrative in comparing rates of P-450-dependent NADPH oxidation in the absence of substrate. NADPH oxidation proceeded at a rate more than three times higher when microsomal phospholipids were used, i.e., at the same rate as obtained in the dilauroylphosphatidylcholine system. Thus, the electron transfer from NADPH to cytochrome $P\text{-}450_{LM_2}$ is probably inefficient in the egg yolk phosphatidylcholine vesicles probably because of nonoptimal interactions between P-450 and P-450 reductase. The reason for this difference is presently unknown, but our preliminary experiments indicate that the electron flow to $P\text{-}450_{LM_2}$ and thus the hydroxylase activity are dependent on the charge of the phospholipids of the membrane double layer.[3] Accordingly, $P\text{-}450_{LM_2}$ is a more efficient catalyst when the protein is incorporated into a more negatively charged membrane, i.e., when phospholipids such as phosphatidylethanolamine and phosphatidylserine are also present in the vesicles.

Interestingly, the substrate specificity of cytochrome $P\text{-}450_{LM_2}$ changed upon incorporation of the protein into the vesicles prepared from microsomal phospholipids while the specificity in egg yolk phosphatidylcholine vesicles and the dilauroylphosphatidylcholine system was essentially the same. Ethylmorphine turned out to be much more efficiently metabolized in the "microsomal" vesicles than in the other reconstituted systems.

A changed substrate specificity upon incorporation of the protein into vesicles was also evident for cytochrome $P\text{-}450_{LM_3}$ (Table I). The most striking differences were obtained with androstenedione, benzo[*a*]pyrene, and 7-ethoxycoumarin as substrates. The steroid was 12 times more efficiently hydroxylated in the egg yolk phosphatidylcholine vesicles than in the dilauroylphosphatidylcholine system, as indicated above, while the situation was essentially reversed for 7-ethoxycoumarin and benzo[*a*]pyrene. As evident from Table II, differences in the substrate-dependent NADPH oxidase activities between the two types of reconstituted systems were also present. The altered hydroxylase activities upon incorporation were often correlated with concomitant changes in the rate of electron transfer from NADPH to $P\text{-}450_{LM_3}$.

$P\text{-}450_{LM_4}$-catalyzed para-hydroxylation of acetanilide proceeded at a rate two times higher in the dilauroylphosphatidylcholine system than in the egg yolk phosphatidylcholine vesicles, and O-deethylation of 7-ethoxycoumarin proceeded at a rate five times higher in the nonmembranous system (Table I). No pronounced differences were seen in NADPH oxidase activity between the two types of reconstituted systems.

From Table III it becomes evident that some substrates (e.g., ethylmorphine and aminopurine) are more efficiently hydroxylated by P-450 than others, i.e., fewer amounts of H_2O_2 are produced when these substrates are bound to P-450. Examples of "ineffective" substrates are androstenedione and benzo[*a*]pyrene. For substrates like androstenedione and 7-ethoxycoumarin, the "effectiveness" in the hydroxylation reaction was markedly different in the two types of reconstituted systems, 7-ethoxycoumarin being more than four times more efficiently metabolized in "microsomal" vesicles containing $P\text{-}450_{LM_2}$ than in the dilauroylphosphatidylcholine system, while in the case of androstenedione as substrate the situation was almost the reverse. This indicates altered properties of the P-450 enzymes upon incorporation into the vesicles.

Our recent experiments[4] indicate that the effectiveness in the hydroxylation reactions is greatly enhanced when cyto-

[3] M. Ingelman-Sundberg, T. Haaparanta, and J. Rydström, unpublished experiments.

[4] M. Ingelman-Sundberg and I. Johansson, unpublished experiments.

Table III: Ratios between Cytochrome P-450 Dependent NADPH Oxidase Activity and Catalytic Activity in Reconstituted Vesicles Prepared from either Microsomal Phospholipids (MIC.P.L.) or Egg Yolk Phosphatidylcholine (EYPC) and in the Dilauroylphosphatidylcholine System (DLPC)[a]

	ratio of NADPH oxidase activity to catalytic activity						
	LM_2			LM_3		LM_4	
substrate	vesicles MIC.P.L.	vesicles EYPC	DLPC	vesicles EYPC	DLPC	vesicles EYPC	DLPC
androstenedione, 350 μM	133	91	25	27	100	*b*	*b*
ethylmorphine, 1.3 mM	1.9	1.9	1	7.4	9.0	*b*	*b*
aminopurine, 2.3 mM	1.0	1.5	1	2.3	1.9	*b*	*b*
7-ethoxycoumarin, 50 μM	17	34	76	14.5	7.5	214	26
benzo[*a*]pyrene, 25 μM	*b*	850	65	213	60	*b*	*b*
acetanilide, 1.3 mM	*b*	*b*	*b*	*b*	*b*	4.6	4.8

[a] The values are taken from Tables I and II. [b] Not determined.

chrome b_5 has been incorporated together with the other proteins into the vesicles at a molar ratio of P-450 to b_5 present in the microsomes. The hydroxylase activities of all three types of P-450 are increased 2–6 times, depending on the substrate used. The enhanced hydroxylase activity is correlated to a diminished production of O_2^- and H_2O_2 in the system, thereby indicating that cytochrome b_5 is a more efficient donor of the second electron to cytochrome P-450 than is NADPH–cytochrome P-450 reductase. No effects of b_5 were seen when using the nonmembranous dilauroylphosphatidylcholine system.

Conclusions

Pronounced changes in the catalytic properties of cytochromes $P\text{-}450_{LM_2}$ and $P\text{-}450_{LM_3}$ were obtained upon incorporation of the proteins into phospholipid vesicles. Kinetics possibly indicative of positive cooperativity were obtained for androstenedione hydroxylation in microsomes and in $P\text{-}450_{LM_3}$-containing vesicles but could not be reconstituted when using the dilauroylphosphatidylcholine system. A possible explanation is that in contrast to the situation in the membranous vesicles, protein cluster formation is not possible in the dilauroylphosphatidylcholine system. Our preliminary cross-linking experiments support this view.[5]

There may be at least three different explanations for the different catalytic properties of the membrane-bound P-450 proteins. (1) Changes in the catalytic properties were often correlated with concomitant changes in NADPH oxidase activity. Therefore, it is likely that facilatated interactions between NADPH–cytochrome P-450 reductase and cytochrome P-450 in some cases give rise to increased hydroxylase activity. (2) When hydrophobic substrates and membranous systems are used, the binding of the substrate to the enzyme and thus hydroxylase activity are facilitated (Narasimhulu, 1977). (3) Since some substrates used are water soluble and, furthermore, as differential effects were seen between $P\text{-}450_{LM_2}$ and $P\text{-}450_{LM_3}$ upon incorporation of the proteins into the vesicles when the same series of substrates are used, it seems likely that some of the effects are attributed to the direct action of the membranous phospholipids on the catalytic properties of the P-450 molecule itself.

In summary, the membranous system for reconstitution of the liver microsomal hydroxylase system has been shown to exhibit different properties than the dilauroylphosphatidylcholine system. Our experiments with cytochrome b_5[4] indicate that there are additional factors other than a native membrane structure that have to be introduced before a complete reconstitution of the liver microsomal hydroxylase system has been achieved.

[5] A.-L. Edvardsson and M. Ingelman-Sundberg, unpublished experiments.

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Characterization of the Histidine Residues in Alkaline Phosphatase by Carbon-13 Nuclear Magnetic Resonance†

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ABSTRACT: β,β-[γ-^{13}C]Dideuteriohistidine has been biosynthetically incorporated into alkaline phosphatase from *Escherichia coli* and utilized as a nonperturbing ^{13}C nuclear magnetic resonance (NMR) probe of the environments of the histidine residues in this zinc metalloenzyme. The ^{13}C NMR spectrum of the labeled enzyme exhibits 9 separate resonances arising from the 10 histidine residues located in each of the symmetrically disposed subunits of the dimer. The excellent resolution and large chemical shift range (14 ppm) displayed by these signals are direct consequences of the sensitivity of the histidine γ-carbon chemical shift to the ionization state and tautomeric form of the imidazole side chains and the coordination of several of these to metal ion. The environments of the individual histidine residues were inferred by investigating the chemical shift responses of their ^{13}C resonances to enzyme metal composition, pH, and inhibitor binding. Additional information concerning their motional freedom was obtained from spin relaxation measurements which were analyzed in terms of the contributions expected from intramolecular ^{13}C–^{1}H and ^{13}C–^{14}N dipolar relaxation and chemical shift anisotropy. The combined results indicate that 4 of the 10 histidines, the only ones that titrate with pH, are surface residues located relatively remote from the active site. Of the six nontitrating residues, one appears to be buried in a solvent-inaccessible region of the protein. Three others are almost certainly involved in metal ion ligation to active-site metal ion(s), two via their N^{π} nitrogen atoms and the other via N^{τ}. The spectral characteristics of the remaining two histidine residues strongly suggest they are also located at or near the active site. One or both may also participate in metal ion coordination, although the current evidence for this is inconclusive.

Escherichia coli alkaline phosphatase is a dimeric zinc metalloenzyme that has the ability to hydrolyze a broad spectrum of phosphate monoesters with little variation in rate. As the result of numerous investigations utilizing a wide variety of techniques, the mechanism by which this broad specificity is achieved is now well understood (Reid & Wilson, 1971; Coleman & Chlebowski, 1979). At the same time, however, conflicting reports have appeared concerning such questions as metal ion stoichiometry, the catalytic roles of the metal ions, and the existence of subunit interactions (negative cooperativity).

Much of the confusion regarding these issues stems from a lack of knowledge concerning the structural features of the enzyme involved in substrate and metal ion binding. At the present time, a high-resolution X-ray structure of alkaline phosphatase is unavailable. Previous work has established the existence of an active-site serine residue, whose covalent phosphorylation constitutes a key reaction intermediate (Schwartz & Lipmann, 1961; Levine et al., 1969), and other data have suggested the involvement of an arginine residue (Daemen & Riordan, 1974) and one or more histidine residues (Tait & Vallee, 1966; Taylor & Coleman, 1972; Csopak & Falk, 1974) in substrate and metal ion binding, respectively. Spectroscopic methods that have been applied to the study of alkaline phosphatase structure have virtually all relied on the replacement of the native group 2B metal ion Zn^{2+}, which is intrinsically a poor spectroscopic probe because of its filled d shell, with transition metal ions such as Co^{2+}, Mn^{2+}, or Cu^{2+} (Coleman & Chlebowski, 1979). These transition-metal probes have provided valuable structural information, but they suffer somewhat from their intrinsically perturbing nature, which arises from differences in their ionic radii, preferred

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