the concentration of inactivator in the cases which are isotopically "mixed" and for the invariance of k_i in the isotopically "homogeneous" cases. Accordingly, we conclude that the mechanism of inactivation, like that of catalysis, involves as the first step abstraction of hydrogen from C-1 of glycerol and transfer of this hydrogen to the 5′-carbon of cofactor. The three hydrogens thereby attached to C-5′ of deoxyadenosine become equivalent and removal of one of them is involved in the rate-determining step for inactivation. Whether this transfer from 5′-deoxyadenosine involves readdition of hydrogen to the glycerol skeleton or transfer to some other group or species associated with the enzyme or coenzyme remains unclear, as does the nature of the inactivated holoenzyme complex. The identification of the nucleoside in the inactivated complex as 5′-deoxyadenosine suggests that the 5′-deoxyadenosyl moiety which obtains after the second hydrogen-transfer step is capable of acquiring a third hydrogen, possibly either from substrate or a nearby amino acid residue, since intact AdoCbl cannot be recovered following glycerol inactivation (Bachovchin et al., 1977).

A 5′-deoxyadenosine moiety containing three equivalent hydrogens has long been considered as an intermediate in catalysis. The significance of these results for the mechanism of catalysis is that they establish kinetically the feasibility of such an intermediate in the mechanism of glycerol inactivation, a mechanism which is very similar to and may in fact be identical with the mechanism of catalysis. The major unique feature of inactivation could be that enzyme-bound "product" formed in the normal way from glycerol bound in an "EG_S" fashion effects irreversible inactivation of the holoenzyme complex.

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Studies on the Microsomal Mixed Function Oxidase System: Redox Properties of Detergent-Solubilized NADPH-Cytochrome P-450 Reductase†

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ABSTRACT: Hepatic microsomal NADPH-cytochrome P-450 reductase was solubilized from rabbit liver microsomes in the presence of detergents and purified to homogeneity by column chromatography. The purified reductase had a molecular weight of 78 000 and contained 1 mol each of FAD and FMN per mol of enzyme. On reduction with NADPH in the presence of molecular oxygen, an O_2-stable semiquinone containing one flavin free radical per two flavins was formed, in agreement with previous work on purified trypsin-solubilized reductase. The reduction of oxidized enzyme by NADPH, and autoxidation of NADPH-reduced enzyme by air, proceeded by both one-electron equivalent and two-electron equivalent mechanisms. The reductase reduced cytochrome P-450 (from phenobarbital-treated rabbits) and cytochrome P-448 (from 3-methylcholanthrene-treated rabbits). The rate of reduction of cytochrome P-450 increased in the presence of a substrate, benzphetamine, but that of cytochrome P-448 did not.

Hepatic NADPH-cytochrome P-450 reductase (EC 1.6.2.4), the flavoprotein component of a liver microsomal mixed-function oxidase, contains 1 mol each of FAD and FMN per mol of enzyme. The two flavins have different properties, e.g., oxidation–reduction characteristics (Iyanagi & Mason, 1973; Iyanagi et al., 1974). An active mixed-function oxidase system can be reconstituted from microsomal fractions containing the NADPH-cytochrome P-450 reductase, cytochrome P-450, and phosphatidylcholine (Lu & Coon, 1968). These components have been purified in several laboratories. The purified detergent-solubilized NADPH-cytochrome P-450 reductase, which also contains one molecule each of FAD and FMN, can reduce cytochrome P-450 directly in a reconstituted

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system (Vermilion & Coon, 1974; Yasukochi & Masters, 1976; Dignam & Strobel, 1977).

In order to understand the mechanism of electron transfer between the reductase and cytochrome P-450, we have studied the oxidation-reduction characteristics of detergent-solubilized cytochrome P-450 reductase. In this work, the NADPH-cytochrome P-450 reductase was purified with detergents from rabbit liver microsomes by the method of Iyanagi & Mason (1973) with some modifications. The properties of this enzyme were compared with those of the trypsin-solubilized enzyme. The reactivities of the reductase with cytochromes P-450 and P-448 were studied in the reconstituted system with stopped-flow technique.

Experimental Procedures

Methods. Optical spectra were measured with a Shimazu Model UV-200 spectrophotometer, in a sample compartment thermostated at 25 °C. EPR[1] derivative absorption spectra were observed with a JEOL Model JES-FX spectrometer. The EPR spectra at room temperature were recorded in collaboration with Dr. Masahiro Kohno at JEOL Ltd., using a flat cell. Fluorescence measurements for flavin determination using phosphodiesterase (Iyanagi & Mason, 1973) were made with a Hitachi Model 204-S spectrophotometer. Benzphetamine N-demethylation activity was assayed by measuring benzphetamine-dependent NADPH oxidation (at 340 nm) by a modification of the method of Lu et al. (1970). Cytochrome P-450, NADPH-cytochrome P-450 reductase, and dilauroylphosphatidylcholine were preincubated for 5 min at the concentrations indicated in the legend of Figure 6 (final concentrations) and then diluted with 0.10 M potassium phosphate buffer, pH 7.25. NADPH-cytochrome *c* reductase activity was assayed by the method of Imai (1976).

Stopped-flow experiments were performed by using a Union Giken Model RA-401 stopped-flow spectrophotometer equipped with a kinetic data processor (RA-456) and a Yokogawa Type 3083 recorder. The rate of cytochrome P-450 or P-448 reduction was determined by following the formation of the CO complex of ferrous cytochrome P-450. In one syringe of the stopped-flow apparatus was placed a solution containing 0.2 μM cytochrome P-450 (or cytochrome P-448), 0.4 μM (as flavin) NADPH-cytochrome P-450 reductase, 1 mM benzphetamine, and 0.1 M potassium phosphate buffer, pH 7.25. The cytochrome, the reductase, and dilauroylphosphatidylcholine were preincubated for 5 min at room temperature before dilution with the buffer, and the whole solution was saturated with CO by thorough bubbling. In the other syringe of the apparatus was placed a CO-saturated solution containing 200 μM NADPH, 1 mM benzphetamine, and 0.1 M potassium phosphate buffer, pH 7.25. Equal volumes of the two solutions were mixed in the mixing chamber (dead time about 2 ms) and the increase in absorbance at 450 nm (or 448 nm) was recorded.

Cytochrome P-450 and P-448 concentrations were determined from the difference spectrum, reduced CO complex minus reduced, $\Delta\epsilon_{450\text{-}490nm} = 91$ mM^{-1} cm^{-1} (Omura & Sato, 1964) and $\Delta\epsilon_{448\text{-}490nm} = 91$ mM^{-1} cm^{-1}, respectively. FAD and FMN concentrations were determined spectrophotometrically using $\epsilon_{450nm} = 11.3$ mM^{-1} cm^{-1} and $\epsilon_{445nm} = 12.5$ mM^{-1} cm^{-1} at pH 7.0 (Koziol, 1971), respectively. The extinction coefficient for detergent-solubilized NADPH-cytochrome P-450 reductase was found to be 11.5 mM^{-1} cm^{-1} at 455 nm by the method of Mayhew & Massey (1969).

Preparation of Microsomes. Rabbits (about 2.5 kg) were given intraperitoneal injections of 50 mg of sodium phenobarbital/kg of body weight, each day for 7 days. The livers were perfused with 0.15 M KCl and then homogenized with 4 volumes of ice-cold 0.15 M KCl containing 10 mM EDTA (adjusted to pH 7.25 with sodium hydroxide), using a Matsushita homogenizer, Model MX-140 S, for 2 min. The homogenate was centrifuged at 10 000*g* for 20 min; the supernatant solution was then centrifuged at 23 000 rpm (Hitachi barrel rotor, RPB, $g_{max} = 55\,000g$) for 120 min. The sediment was resuspended in 0.15 M KCl containing 10 mM EDTA and recentrifuged at the same speed, to give the microsomal fraction, which was stored in water suspension at −70 °C.

Preparation of NADPH-Cytochrome P-450 Reductase from Trypsin-Treated Microsomes. Rabbit trypsin-solubilized NADPH-cytochrome P-450 reductase was prepared by the method of Iyanagi & Mason (1973) with some modifications (Omura & Takesue, 1970).

Solubilization and Purification of NADPH-cytochrome P-450 Reductase. Detergent-solubilized NADPH-cytochrome P-450 reductase was prepared by the method of Iyanagi & Mason (1973) with some modifications. A suspension of microsomes, about 8 g of protein in 1 L of 0.1 M Tris-acetate buffer, pH 7.6, was stirred for 30 min at 0 °C with 200 mL of glycerol, 446 mg of EDTA, 5 g of deoxycholate, 25 mL of Triton N-101, and 6.5 g of KCl; the precipitate recovered by centrifugation at 78 000*g* for 120 min was discarded. The supernatant solution was applied to a DEAE-cellulose (DE-22) column (3.0 × 20 cm) previously equilibrated with 0.1 M Tris-acetate, pH 7.6, containing 20% glycerol, 0.1% Triton N-101, and 1 mM EDTA (buffer A) containing 0.075 M KCl, to remove cytochrome P-450 and cytochrome b_5, but not reductase. The reductase was then eluted with buffer A containing 0.5 M KCl. The active fraction was diluted tenfold with buffer A and applied to a second DEAE-cellulose column previously equilibrated with buffer A, and eluted with a linear gradient, 0 to 0.5 M KCl in buffer A. The active fractions were dialyzed overnight against 2 L of 25 mM of potassium phosphate buffer, pH 7.0, containing 20% glycerol, and 0.1% Triton N-101 (25 mM buffer B), and applied to a hydroxylapatite column (3.0 × 15 cm) equilibrated with 25 mM buffer B; the column was washed with 200 mL of 40 mM buffer B and the reductase was then eluted with 75 mM buffer B. The active yellow fraction was diluted twofold with 20% glycerol containing 0.1% Triton N-101 and applied to a second hydroxylapatite column (1.5 × 15 cm) equilibrated with 25 mM buffer B and then eluted with 100 mM buffer B. The bright yellow fraction was applied to a DEAE-Sephadex A-50 column (1.5 × 15 cm) equilibrated with 100 mM buffer B. The column was washed with 200 mL of 100 mM buffer B containing 0.1 M KCl, then eluted with 100 mM buffer B containing 0.5 M KCl. The active fraction was diluted tenfold with 20% glycerol containing 0.1% Triton N-101 and applied to a third hydroxylapatite column (1.5 × 15 cm) equilibrated with 25 mM potassium phosphate buffer containing 20% glycerol. The column was washed with 100 mL of 50 mM potassium phosphate buffer containing 20% glycerol and the reductase was then eluted with 200 mL of potassium phosphate buffer containing 20% glycerol. The yield of the purified reductase was about 15 mg from about 8 g of microsomes. The active fraction had a specific activity of 35 μmol min^{-1} mg^{-1} at 25 °C (cytochrome *c* reduction). The enzyme contained 20.7 nmol of total fla-

[1] Abbreviations used: EPR, electron paramagnetic resonance. For purposes of discussion, FAD, FADH, $FADH_2$, FMN, FMNH, and $FMNH_2$ are used to symbolize the flavin species: FlH, oxidized flavin; FlH_2, neutral flavoprotein semiquinone; and FlH_3, neutral fully reduced flavoprotein (Hemmerich et al., 1965). Where the individual species of flavins are not known but are relevant to the discussion, they are referred to as F_l, $F_lH\cdot$, F_lH_2, F_2, $F_2H\cdot$, and F_2H_2.

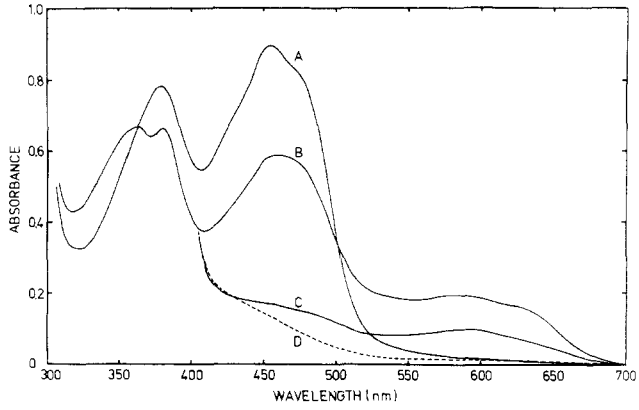

FIGURE 1: Absorption spectra of detergent-solubilized NADPH-cytochrome P-450 reductase. The O_2-stable reductase semiquinone was prepared by adding NADPH (156 μM, final concentration) in the presence of air to enzyme (78.0 μM) in 0.1 M potassium phosphate buffer, pH 7.0, containing 20% glycerol. The system was allowed to stand for about 15 min. (Curve A) Oxidized; (curve B) O_2-stable semiquinone form (O_2-stable semiquinone); (curve C) after additional NADPH (2 mM, final concentration) under anaerobic conditions (NADPH-residual semiquinone); (curve D) hydrosulfite reduced.

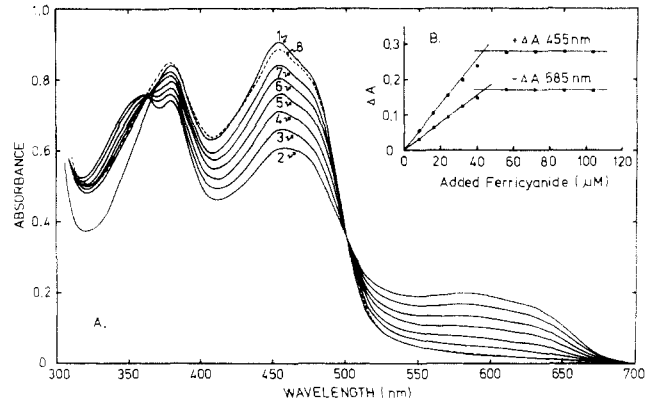

FIGURE 2: (A) Stepwise oxidation of the O_2-stable semiquinone of detergent-solubilized NADPH-cytochrome P-450 reductase by ferricyanide. The O_2-stable semiquinone was prepared by adding NADPH (160 μM, final concentration) in the presence of air to enzyme (78.6 μM), and the system was allowed to stand to 20 min. (A) The O_2-stable semiquinone reductase (78.6 μM) in 0.1 M potassium phosphate buffer, pH 7.0, containing 20% glycerol was titrated with 2 mM potassium ferricyanide in 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C. (Curve 1) Fully oxidized enzyme; (curve 2) O_2-stable semiquinone form; (curves 3–8) equilibrium absorption spectra after the addition of 8, 16, 24, 32, 40, 56 μM ferricyanide, respectively. The dashed line is that of curve 8. The spectra shown were not corrected for the slight dilution, less than 5%, which occurred during the course of the titration. The inset (B) shows the changes occurring at 455 and 585 nm during the titration with ferricyanide.

vin/mg of protein; the ratio of FMN to FAD was 0.95. The molecular weight was 78 000 (gel electrophoresis in the presence of sodium dodecyl sulfate).

Preparation of Cytochrome P-450 and Cytochrome P-448. A homogeneous preparation of cytochrome P-450 was prepared by the method of Imai & Sato (1974) from liver microsomes of phenobarbital-treated rabbits. The high-spin form of cytochrome P-448 was purified by the method of Hashimoto & Imai (1976) from 3-methylcholanthrene-induced rabbit liver microsomes. The cytochrome P-450 and cytochrome P-448 used in this study contained 16.8 and 17.1 nmol of hemoprotein/mg of protein, respectively.

Materials. NADPH was purchased from the Oriental Yeast Co., cytochrome *c* from Boehringer, Triton N-101 (alkylphenoxypolyethoxyethanol) and dilauroylphosphatidylcholine were from Sigma, DEAE-Sephadex A-50 and CM-Sephadex C-50 were from Pharmacia, and DE-22 was from Whatman Ltd. Benzphetamine hydrochloride was a gift from Dr. K. Kamataki of Chiba University. Hydroxylapatite was prepared by the method of Lovin (1955). All other reagents were of the highest grade available commercially.

Results

Absorption Spectra of Detergent-Solubilized NADPH-Cytochrome P-450 Reductase. The visible absorption spectrum of purified NADPH-cytochrome P-450 reductase at several oxidation levels is shown in Figure 1. The oxidized enzyme has absorption peaks at 380 and 455 mm and a shoulder at 476 nm, typical of a flavoprotein. The O_2-stable semiquinone was obtained by reduction with NADPH in the presence of oxygen. The spectrum so obtained (Figure 1, curve B) was very similar to that of the O_2-stable semiquinone of the trypsin-solubilized enzyme (Iyanagi & Mason, 1973). The O_2-stable semiquinone form was not completely reduced by excess NADPH (Figure 1, curve C). The detergent-solubilized NADPH-cytochrome P-450 reductase thus exhibits the same spectral properties as the trypsin-solubilized enzyme (Iyanagi & Mason, 1973).

Oxidation Level of the O_2-Stable Semiquinone. The O_2-stable semiquinone was titrated with aliquots of potassium ferricyanide. The successive absorption spectra obtained, and the relationship between absorbance changes at 455 and 585 nm and the amount of ferricyanide added, are shown in Figure 2. The maximum increase at 455 nm and maximum decrease at 585 nm were observed when 1.1 mol of ferricyanide had been added per 2 mol of flavin, that is, 1 oxidizing equiv/2 flavins, confirming our previous observation that *the O_2-stable semiquinone of trypsin-solubilized reductase is reduced 1 equiv more than the fully oxidized enzyme* (Iyanagi & Mason, 1973). This result shows that the oxidation level of the O_2-stable semiquinone is not affected by trypsin treatment of the enzyme. Our present results are in agreement with the recent work of Vermilion & Coon (1977), but not with those of Masters and her colleagues (Masters et al., 1975; Yasukochi & Masters, 1976, 1977), and Dignam & Strobel (1977).

EPR Spectra of NADPH-Cytochrome P-450 Reductase. The free radical nature of the O_2-stable semiquinone form of detergent-solubilized enzyme was compared with that from trypsin-solubilized enzyme. The signal heights in the $g = 2.00$ region of the EPR spectra were almost identical (Figure 3B). The extent of the decrease in optical absorption at 585 nm in the presence of excess NADPH (Figure 1, curve C) was also in good agreement with the decrease of signal height as judged by EPR spectrometry (Figure 3A, curve 2). The microwave power dependence of the signal in the presence of excess NADPH is shown in Figure 3B. The NADPH-residual signal,[2] which represents about 40% of the O_2-stable semiquinone, has the same shape as, and saturation characteristics similar to, that of the O_2-stable semiquinone, except that the NADPH-residual signal of detergent-solubilized enzyme is somewhat more saturable than that of trypsin-solubilized enzyme (Figure 3B). Since the spin concentration of the O_2-stable semiquinone of trypsin-solubilized enzyme is about 50% of the total flavin concentration when flavodoxin semiquinone was used as a standard of comparison (Iyanagi & Mason, 1973), these re-

[2] The NADPH-residual semiquinone is a roughly equimolar mixture of 3-electron- and 4-electron-reduced enzyme (Iyanagi et al., 1974). However, it is not clear from the present work whether the residual signal is attributable to the same flavin semiquinone form as is O_2 stable, or to the semiquinone form of the other flavin.

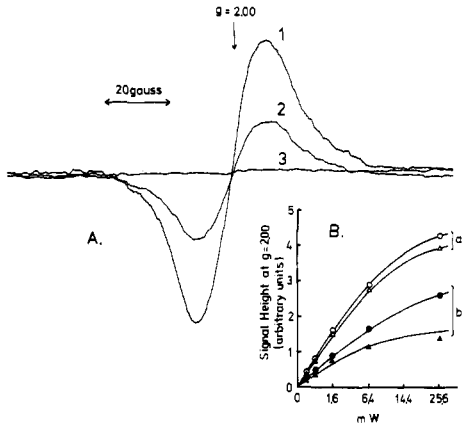

FIGURE 3: Room temperature EPR signals of NADPH-cytochrome P-450 reductase (A) EPR spectrum of detergent-solubilized NADPH-cytochrome P-450 reductase. (Curve 1) The O_2-stable reductase semiquinone (78.0 μM); (curve 2) NADPH-residual semiquinone; (curve 3) oxidized enzyme. Spectra were observed at 8 mW, modulation amplitude, 6.3 G, scanning rate, 50 G/min. The O_2-stable reductase semiquinone (curve 1) and NADPH-residual semiquinone (curve 2) were prepared as described in Figure 1. (B) Microwave power-saturation characteristics of the $g = 2.00$ signal from detergent- and trypsin-solubilized NADPH-cytochrome P-450 reductases. Enzyme concentration and EPR conditions are the same as in A. (Curve a) O_2-stable reductase semiquinone: detergent-solubilized enzyme (▲) and trypsin-solubilized enzyme (○). (Curve b) NADPH-residual semiquinone: detergent-solubilized (▲) and trypsin-solubilized (●).

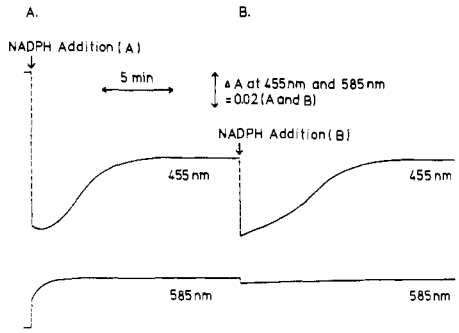

FIGURE 4: The time course of oxidation-reduction reactions of flavins during autoxidation of NADPH-reduced enzyme. Oxidized enzyme (A) or O_2-stable semiquinone form (B) was mixed aerobically with NADPH, and the reduction and subsequent autoxidations followed spectrophotometrically. Concentrations: 13 μM NADPH-cytochrome P-450 reductase, 26 μM NADPH (added at A and again at B) in 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C. Curves show flavin oxidation-reduction at 455 and 585 nm.

sults show that the spin state of the O_2-stable semiquinone is not modified by trypsin treatment, and that the O_2-stable semiquinone form of detergent-solubilized enzyme contains one fully oxidized flavin and one flavin semiquinone.

Reduction of Detergent-Solubilized NADPH-Cytochrome P-450 Reductase with NADPH. The oxidase activity of this enzyme is very slow, with a turnover number of 1 to 2 min^{-1}, and is therefore convenient for studying intermediate forms during the oxidation-reduction reactions. The increases in absorbance at 585 nm and at 455 nm during the oxidation of NADPH-reduced enzyme by air were not parallel. The changes at 585 nm were complete after about 2 min, but those at 455 nm continued (Figure 4A). When the O_2-stable reductase semiquinone was then mixed with NADPH in the presence of air, rapid decrease in absorbance at 455 nm was accompanied by a small change of absorbance at 585 nm, and then air oxidation caused an increase of absorbance at 455 nm with an accompanying small change at 585 nm (Figure 4B).

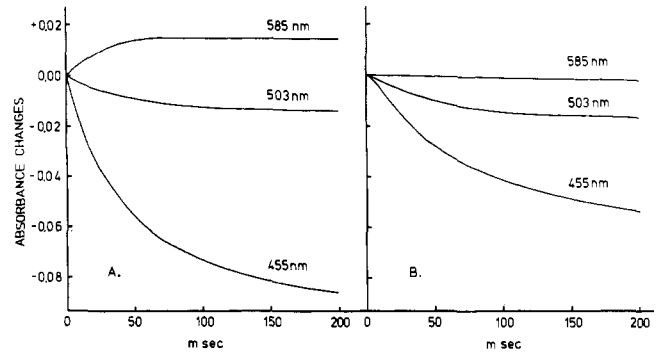

FIGURE 5: Reduction of the enzyme by NADPH. (A) Oxidized enzyme was mixed with NADPH aerobically, and absorbance changes were recorded at three different wavelengths. Final concentrations were: 13 μM NADPH-cytochrome P-450 reductase, 26 μM NADPH, and 0.1 M potassium phosphate buffer, pH 7.0. (B) Reduction of the O_2-stable reductase semiquinone by NADPH. O_2-stable semiquinone was mixed with NADPH aerobically and absorbance changes were recorded at three different wavelengths. Final concentrations were: 13 μM O_2-stable reductase semiquinone, 26 μM NADPH, and 0.1 M potassium phosphate buffer, pH 7.0. Formation of reduced flavin was followed at 503 nm (isosbestic of oxidized and O_2-stable semiquinone), and formation of semiquinone at 585 nm.

Rapid spectrophotometric studies have shown that the changes in absorbance at 455 nm (negative) and 585 nm (positive) during the reduction of oxidized enzyme by NADPH were not parallel. The changes in absorbance at 585 nm were complete after about 75 ms, but those at 455 nm were still not complete after 200 ms (Figure 5A). During the reduction of O_2-stable semiquinone by NADPH, the decrease in absorbance at 455 nm and 585 nm was observed, but the changes at 585 nm were very small (Figure 5B). Even in the presence of a large excess of NADPH (40-fold), the decrease at 585 nm was very small (data not shown). This observation disagrees with static spectrophotometric experiments (Figure 1, curve C). The extent of reduction calculated from the changes at 585 nm (Figure 1, curve C) was only about 10%. However, slow reduction beyond the time scale of Figure 5 occurred in the case of excess NADPH addition. Since almost identical results were obtained in anaerobic conditions, the rapid reduction process by NADPH does not contain detectable effects of air oxidation within 200 ms (Figure 5). In any case, the oxidation-reduction properties of the semiquinone(s) and reduced flavin(s) are clearly different.

Electron Transfer from Detergent-Solubilized NADPH-Cytochrome P-450 Reductase to Cytochrome P-450. When recombined with NADPH-cytochrome P-450 reductase, cytochrome P-450 catalyzed the NADPH-dependent N-demethylation of benzphetamine. As shown in Figure 6A, the addition of phospholipid to the reaction mixture stimulated NADPH-dependent N-demethylation of benzphetamine about two- to threefold. The optimal amount of the phospholipid was about 1 μM (Figure 6A). Slow NADPH oxidation was observed even in the absence of benzphetamine (Figure 6A). Since this reaction was very slow in the absence of cytochrome P-450, NADPH oxidase activity must be due to the cytochrome P-450. Under these conditions (Figure 6A), the molar ratio of cytochrome P-450 to reductase in the mixture was 1:1. The rate of NADPH oxidation in the presence of benzphetamine was approximately linear with increasing concentrations of cytochrome P-450 (Figure 6B) and NADPH-cytochrome P-450 reductase (data not shown). The rate of cytochrome P-450 reduction was determined in the stopped-flow spectrophotometer by following the formation of the CO complex of reduced cytochrome P-450 under the same conditions used in

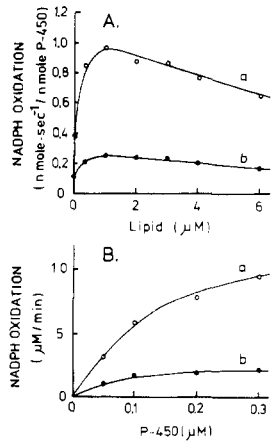

FIGURE 6: Stimulation of benzphetamine N-demethylation by phospholipid. (A) The reaction mixture, in a final volume of 2.5 mL, contained 0.1 M potassium phosphate buffer, pH 7.25, 100 μM NADPH, 0.2 μM NADPH-cytochrome P-450 reductase (as flavin), 0.1 μM cytochrome P-450, and the indicated amounts of phospholipid. (B) The reaction mixture, in a final volume 2.5 mL, contained 0.1 M potassium phosphate buffer, pH 7.25, 100 μM NADPH, 0.2 μM NADPH-cytochrome, P-450 reductase (as flavin), 3 μM phospholipid, 0.0008% Emalgen 913, and the indicated amounts of cytochrome P-450. In both A and B: (curve a) 1 mM benzphetamine present; and (curve b) benzphetamine absent.

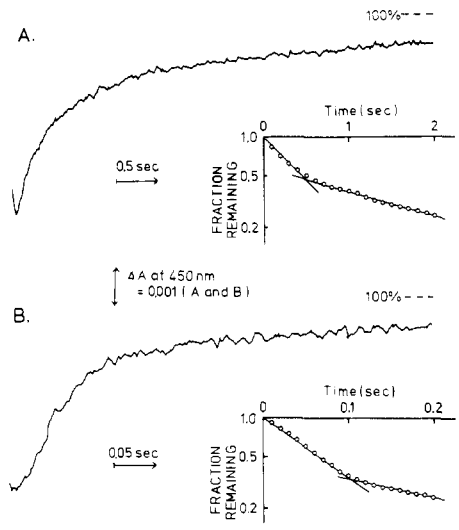

FIGURE 7: Time course of the reduction of cytochrome P-450 (from phenobarbital-treated rabbits) by NADPH in the absence and presence of benzphetamine. The experiments were performed in the stopped-flow spectrophotometer as described in Materials and Methods. The dotted line indicates the final absorbance: (A) in the absence of benzphetamine and (B) in the presence of 1 mM benzphetamine. The insets show first-order plots of the rate of cytochrome P-450 reduction against time (t). $(A_t - A_{max})/A_{max}$ was plotted vs. time, t, where A_t and A_{max} are the changes in absorbance at any time t (seconds) and at the completion of reduction, respectively.

the experiment shown in Figure 6A. As expected from Figure 6A, the reductase reduced cytochrome P-450 even in the absence of benzphetamine, but the rate of cytochrome P-450 reduction was increased about fivefold in its presence (Figure 7), in good agreement with results from a reconstituted system using partially purified rat liver reductase and highly purified rabbit cytochrome P-450 (Imai et al., 1977). In either absence or presence of the substrate, the reaction was clearly biphasic, consisting of two first-order processes (Figure 7), from which the apparent first-order rate constants for the fast and slow phases could be estimated (Table I). In a reconstituted system containing cytochrome P-448 instead of cytochrome P-450,

TABLE I: Rate Constants Determined for the Reduction of Cytochrome P-450 by NADPH and Reductase in the Reconstituted System.[a]

Cytochrome	Benzphetamine Concn (mM)	Rate constant (s^{-1}) Fast phase	Slow phase	Fast component (% of total)
P-450	—	0.49	4.80	40
	1	2.53	16.95	62
P-448	—	0.46	2.30	40
	1	0.51	2.50	64

[a] k_{slow} and k_{fast} for P-450 were calculated from Figure 7. Each value for P-448 was calculated from experiments with conditions corresponding to those of Figure 7 (data not shown). The rate constant for the rapid phase (k_{fast}) was corrected for the slow phase.

the stimulation of cytochrome P-448 reduction by the addition of benzphetamine was not observed (Table I).

Discussion

If microsomal NADPH-cytochrome P-450 reductase is directly involved in the microsomal reduction of cytochrome P-450 during the mixed function oxidase cycle, it is possible that the electron transfer sequence is:

$$\text{NADPH} \rightarrow \text{fp(FAD} \rightleftharpoons \text{FMN)} \rightarrow \text{P-450}$$

An interesting problem, then, is the mechanism of the two electron transfers. We report here that detergent-solubilized NADPH-cytochrome P-450 reductase as well as the trypsin-solubilized form contains FAD and FMN in equimolar quantities and that the oxidation level of the O_2-stable semiquinone is 1 equiv more reduced than its fully oxidized state containing 4 oxidation equiv judged by ferricyanide titration (Figure 1) and by quantitative EPR spectrometry (Figure 3). These results suggest that the oxidation level of the O_2-stable semiquinone has the following structure:

$$\begin{matrix}\text{FAD} & & \text{FADH}\cdot \\ & \text{or} & \\ \text{FMNH}\cdot & & \text{FMN}\end{matrix}$$

Yasukochi & Masters (1976) consider that this species is the two-electron reduced enzyme. There are several possible two-electron reduced states: F_1H_2–F_2, F_1–F_2H_2, or $F_1H\cdot$–$F_2H\cdot$. However, present and prior work (Iyanagi et al., 1973, 1974; Vermilion & Coon, 1977) rule out these structures. The reason for the difference is not resolved by the present work. Presumably it lies in differences of experimental design and conditions.

The O_2-stable semiquinone form is not completely reduced by excess NADPH, but the extent of optical absorption decrease at 585 nm in the presence of excess NADPH under anaerobic conditions is in good agreement with the decrease of spin concentration (Figures 1 and 3). These results show that the change in absorbance at 585 nm is only that of free radical species. When the O_2-stable semiquinone was mixed with NADPH in the presence of air, the decrease of absorbance at 455 nm followed a small change of absorbance at 585 nm (Figure 4B), indicating the reduction of the oxidized flavin molecule contained in the O_2-stable semiquinone form. This was confirmed by the decrease of absorbance at 503 nm (Figure 5B), where oxidized and O_2-stable semiquinone are isosbestic (Figure 2). The differences in the changes at 585 nm in the static experiments (Figure 1, curve C) and the rapid reduction experiments with excess NADPH may be attributed to the property of the oxidized flavin molecule contained in the O_2-stable semiquinone ($F_1H\cdot$–F_2 or F_1–$F_2H\cdot$) to accept two

electrons from NADPH. The four-electron reduced enzyme (F_1H_2–F_2H_2) may be produced by electron transfer between two three-electron reduced enzyme molecules ($F_1H\cdot$–F_2H_2 or F_1H_2–$F_2H\cdot$).

When the oxidized enzyme was mixed with NADPH, the formation of semiquinone and fully reduced species of flavin occurred independently (Figure 5A). These results are very similar to those obtained by Masters et al. (1965). Kamin et al. (1965) proposed from the kinetics of enzyme reduction that the flavins were not independent but rather interacted with one another (Masters et al., 1965; Kamin et al., 1965). However, the two flavins contained in the enzyme are structurally different and have quite different redox properties (Iyanagi et al., 1974). On these grounds, the simple shuttle mechanism (cycling between two- and four-electron reduced) for the enzyme proposed by Masters & co-workers (1965) is improbable. Finally, the process of oxidation of the NADPH-reduced enzyme showed biphasic oxidation-reduction characteristics, as discussed above, which appear to involve both 1-equiv and 2-equiv oxidation-reduction during the NADPH-reduction and O_2-reoxidation cycle of the enzyme. Since the O_2-stable semiquinone containing 1 reducing equiv per 2 flavins (4 equiv) is inactive toward several electron acceptors such as cytochrome *c*, and since two reducing equivalents must be transferred from NADPH to O_2 during the mixed function oxidase cycle, FAD-FMN pairs may cycle between the one-electron and three-electron forms during the catalytic cycle. The cytochrome P-450 itself is a one-electron acceptor (Peterson et al., 1977), but the mixed function oxidase process requires 2-electron equiv, which are probably transferred separately. Mechanisms for this have been discussed by Iyanagi et al. (1974).

The mixed function oxidase system of liver microsomes has been resolved into three fractions consisting of cytochrome P-450, NADPH-cytochrome P-450 reductase, and a lipid component (Lu & Coon, 1968). Using the present highly purified NADPH-cytochrome P-450 reductase, electron transport from NADPH to cytochrome P-450 was studied in the reconstituted system. Benzphetamine was used as a model substrate (Imai et al., 1977). The reconstituted system required all three components for maximum enzyme activity (Figure 6A). The rates of electron transfer from NADPH-cytochrome P-450 reductase to ferric cytochrome P-450 were measured under conditions similar to those in Figure 6A using stopped-flow methods. Under optimal conditions, the rate of benzphetamine-dependent oxidation of NADPH was about 60 nmol per min per nmol of cytochrome P-450. The reduction of cytochrome P-450 in the absence of benzphetamine proceeded at a rate of 4.80 s^{-1} (k_{fast}), but it increased to 16.95 s^{-1} (k_{fast}) in the presence of the substrate (Table I). The cytochrome P-450–substrate complex appears to be more reducible than substrate-free cytochrome P-450. Benzphetamine binding to cytochrome P-450 causes the spin state equilibrium to shift toward the high-spin form to cytochrome P-450 (Rein et al., 1977). If this change is important for the energy-consuming process of heme-iron reduction, the high-spin cytochrome P-448 (from 3-methylcholanthrene-treated rabbits) should be more reducible than cytochrome P-450 (from phenobarbital-treated rabbits) in the reconstituted system. However, the rate of (high-spin) cytochrome P-448 reduction was nearly equal to that of (low-spin) cytochrome P-450 reduction in the absence of a substrate, benzphetamine (Table I). These results suggest that the high-spin state is not more readily reducible than the low-spin state, although cytochrome P-450 and P-448 are different species, but that the formation of the substrate–cytochrome P-450 complex containing a change of spin state is important for the initial redox reaction of cytochrome P-450. In the case of cytochrome $P\text{-}450_{cam}$ (from *Pseudomonas putida*), the shift to the high-spin state upon substrate binding is dramatic (Tsai et al., 1970), but liver cytochrome P-450 (from phenobarbital-treated rabbits) is in a thermal equilibrium between high- and low-spin states (Rein et al., 1977). This may indicate that substrate binding to liver cytochrome P-450 is not specific, which would be convenient for oxidative metabolism of a wide variety of xenobiotics. The rate of direct cytochrome P-450 reduction in the presence of substrate was faster than that of overall benzphetamine-dependent NADPH oxidation (Figures 6 and 7), which is consistent with the behavior of a reconstituted system consisting of partially purified reductase and purified cytochrome P-450 (Imai et al., 1977).

In summary, the redox properties of detergent purified NADPH-cytochrome P-450 reductase from rabbit liver are very similar to those of the trypsin-solubilized enzyme. However, only the detergent-solubilized reductase was active toward cytochrome P-450. The enzyme contains two flavins, FAD and FMN, in equimolar quantities. They have different redox properties. Accordingly, we suggest that each flavin has an individual function, e.g., one flavin accepts 2 reducing equiv from NADPH (dehydrogenase flavin) and the other acts as a one-electron carrier (flavodoxin-type flavin) for the net two-electron transfer from NADPH to cytochrome P-450. A more detailed kinetic study of two flavins in the reductase will yield useful information about the electron transfer mechanism from NADPH to cytochrome P-450.

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Differential Effects of Prostaglandin Synthetase Stimulators on Inhibition of Cyclooxygenase†

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ABSTRACT: The different effects of prostaglandin synthetase stimulators on inhibition of the cyclooxygenase by structurally distinct classes of nonsteroidal antiinflammatory agents suggest that the enzyme is altered by interaction with these stimulators. Reversible stimulation of prostaglandin synthetase activity by phenols and some other compounds and the relative influence of these stimulators on inhibitors of the cyclooxygenase were determined quantitatively. Two distinct classes of inhibitors were established. The fenamates were relatively weak inhibitors alone but were much more potent in the presence of phenolic compounds. In contrast, ibuprofen, indomethacin, and flurbiprofen were more potent than the fenamates and were reduced in effectiveness by the stimulators, as expected on the basis of two opposing actions. The relative potency of the cyclooxygenase stimulators (phenol > norepinephrine > tryptophan > benzoquinone > anisole) paralleled their synergistic action on the fenamates and their antagonist action on the nonfenamates. This correlation suggests that an enzyme alteration which leads to cyclooxygenase stimulation may also result in increased sensitivity to fenamates and decreased sensitivity to the other inhibitors, possibly by altering their capacity to bind.

Several structurally distinct classes of nonsteroidal antiinflammatory agents are known to inhibit prostaglandin synthetase (EC 1.14.99.1) activity (Vane, 1971; Ham et al., 1972; Gryglewski, 1974). These agents vary from the structural simplicity of aspirin to the complexity of indomethacin and *N*-phenylanthranilic acid analogues. Most of these inhibitors elicit their effect on the cyclooxygenase (Smith & Lands, 1971; Lands et al., 1973; Egan et al., 1976a), the primary enzyme of the prostaglandin synthetase complex. Although their precise mechanism of action is not understood, several of these agents are time dependent (Rome & Lands, 1976) and aspirin acetylates the cyclooxygenase (Roth et al., 1975). Some enzymes other than prostaglandin synthetase are also inhibited by nonsteroidal antiinflammatory agents (Flower & Vane, 1974; Oyanagui, 1976) and in some instances the inhibitors show differential activity toward these enzymes (Zwarenstein et al., 1976).

In contrast, several compounds such as phenols, quinones, heme-containing proteins, and tryptophan have been shown to stimulate prostaglandin synthetase (Nugteren et al., 1966; Takeguchi et al., 1971; Yoshimoto et al., 1970; Miyamoto et al., 1974; Humes et al., 1976). A mechanism for the stimulatory action of phenols as radical scavengers has been proposed (Egan et al., 1976b). Previously, inhibition of the cyclooxygenase had been studied almost exclusively using an acetone-powder enzyme preparation which required the presence of these compounds for activity (Smith & Lands, 1972). Consequently, it was not possible to examine independently the effects of such stimulators on the action of the various prostaglandin synthetase inhibitors.

This manuscript describes the influence of prostaglandin synthetase stimulators on inhibition of the cyclooxygenase by nonsteroidal antiinflammatory agents. The actions of enzyme stimulators on inhibition by the several structurally distinct classes of these inhibitory agents suggest stimulator-induced alterations of the cyclooxygenase molecule.

Experimental Procedure

Materials

Ram seminal vesicles were obtained from a local slaughterhouse and were stored at −70 °C. Arachidonic acid was purchased from P-L Biochemicals Inc. and [1-^{14}C]arachidonic acid (58 mCi/mmol) was obtained from Dhom Products Ltd. Indomethacin, flufenamic acid, meclofenamic acid, mefenamic acid, and niflumic acid were supplied by Merck & Co. Ibuprofen and flurbiprofen were obtained from the Upjohn Co. Silica gel GF thin-layer chromatography plates were purchased from Analtech Inc. New England Nuclear supplied the Liquifluor. All other chemicals and materials were purchased from standard suppliers.

Methods

Preparation of Microsomal Prostaglandin Synthetase. The prostaglandin synthetase enzyme complex from ram seminal

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