Registry No. TyrTS, 9023-45-4; ATP, 56-65-5; Tyr, 60-18-4; Cys, 52-90-4; Gly, 56-40-6.

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Possible Association of NADPH–Cytochrome P-450 Reductase and Cytochrome P-450 in Reconstituted Phospholipid Vesicles†

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ABSTRACT: A fluorescent probe, *N*-(1-anilinonaphth-4-yl)-maleimide (ANM), was specifically labeled to SH group(s) in the hydrophilic moiety of NADPH–cytochrome P-450 reductase at a ratio of 1 ± 0.1 ANM/mol of protein. The ANM-labeled reductase and P-450 were reconstituted in phosphatidylcholine–phosphatidylethanolamine–phosphatidylserine vesicles in which all of the enzymes were functionally active. The reconstitution of the mixed-function oxidase system was found to be strongly dependent on both the lipid to protein molar ratio and phospholipid composition. The interactions of ANM-labeled reductase with P-450 in proteoliposomes were investigated by perturbation of the fluorescence of ANM. Upon incorporation of P-450 into the phospholipids vesicles (ANM-reductase/P-450/lipids ≡ 1:1.4:800), a significant decrease of total fluorescence intensity and slight increase of emission anisotropy of ANM were observed. In the average fluorescence lifetime of ANM bound with reductase, an appreciable change was shown between the absence and presence of P-450 in the vesicles. These data provide clear evidence that significant molecular interactions occur between the two proteins in a membranous reconstituted system.

Both NADPH–cytochrome P-450 reductase and P-450[1] are essential components of the hepatic microsomal mixed-function oxidase system catalyzing the oxidative metabolism of endogenous substrates such as fatty acids, prostaglandins, and steroids as well as many xenobiotics such as drugs, petroleum

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[1] Abbreviations: DLPC, dilauroylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PE, bovine brain phosphatidylethanolamine; PS, bovine brain phosphatidylserine; ANM, *N*-(1-anilinonaphth-4-yl)maleimide; DPH, 1,6-diphenyl-1,3,5-hexatriene; pCMB, *p*-(chloromercuri)-benzoate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ANM-reductase, ANM-labeled NADPH–cytochrome P-450 reductase; P-450, the cytochrome P-450LM_2 induced in rabbit liver by phenobarbital.

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products, and carcinogens (Estabrook et al., 1979; White & Coon, 1980; Omura, 1978; Black & Coon, 1982). NADPH–cytochrome P-450 reductase is anchored to the membrane of the endoplasmic reticulum via a small (M_r 6000–10000) hydrophobic segment (Gum & Strobel, 1979; Black et al., 1979). The large hydrophilic moiety, which contains 1 mol each of FAD and FMN and accepts electrons from NADPH, protrudes from the membrane into the cytoplasmic space. The distinguishable feature of FAD and FMN-binding domains has also been demonstrated on the basis of experiments with chemical modification (Nisimoto & Shibata, 1981, 1982). The P-450, on the other hand, is deeply embedded into the membrane and is believed to accept the first electron from NADPH–cytochrome P-450 reductase and the second electron from cytochrome b_5 (DePierre & Ernster, 1977; Hildebrandt & Estabrook, 1971; Sugiyama et al., 1979). The arrangement of the two enzymes in the membrane has given us questions as to the mechanism of electron transfer from the flavoprotein to the cytochrome and the functional interactions of the proteins in the mixed-function oxidase system. Essentially, two possible mechanisms are conceivable for functional interactions among component proteins of membrane-bound multienzyme system. Thus, it is possible that the interactions are effected by the lateral motion and subsequent collision of the proteins on the plane of membranes (Taniguchi et al., 1979; Daily & Strittmatter, 1980; Yang, 1975; Dean & Gray, 1982), whereas another possibility is that the enzymes exist in membranes as functional clusters in which the interactions take place directly (French et al., 1980; Baskin & Yang, 1980).

Rotational mobility of P-450 was recently demonstrated in microsomal membranes by transient dichroism technique (Richter et al., 1979; McIntosh et al., 1980) and in reconstituted lipid vesicles by delayed fluorescence polarization technique (Greinert et al., 1979) or by magnetic CD spectrum (Bösterling & Trudell, 1982). Since protein rotation is particularly sensitive to protein–protein interactions (Nigg & Cherry, 1980; Cherry & Godfrey, 1981), flash-induced absorption anisotropy techniques of the heme–CO complex has been successfully applied to investigate intermolecular interactions of P-450 with P-450 or with NADPH–cytochrome P-450 reductase in both microsomal membranes and reconstituted vesicles (Richter et al., 1979; Kawato et al., 1982; Gut et al., 1982). The results led to a better understanding of protein–protein interactions of the components of the hepatic microsomal mixed-function oxidase system.

In the present study, by use of the fluorescence intensity and polarization of ANM labeled to NADPH–cytochrome P-450 reductase, the interactions between P-450 and reductase were studied in a vesicle-reconstituted system. This reconstituted system contains sufficient phospholipids that no protein–protein interactions would be induced solely to prevent exposure of hydrophobic protein surfaces to the aqueous environment. The bilayer of these vesicles is composed of a 1:0.5:0.1 mixture of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine which was shown to provide functional and structural similarity to microsomes (Bösterling et al., 1979; Stier et al., 1978).

Materials and Methods

Materials. NADPH, FAD, FMN, Triton N-101, 2′-AMP, and dilauroylphosphatidylcholine (DLPC) were purchased from Sigma, and DEAE-Sepharose CL-6B and 2′,5′-ADP–Sepharose 4B were obtained from Pharmacia. Dioleoylphosphatidylcholine (DOPC, synthetic) was purchased from Nihonshouji, and bovine brain L-α-phosphatidylethanolamine (PE) and bovine brain L-α-phosphatidylserine (PS) were from Calbiochem-Behring. The determination of FMN and FAD concentrations in mixtures of the two compounds was performed at both pH 7.7 and pH 2.6 by a rapid fluorometric method (Faeder & Siegel, 1973).

Solubilization and Purification of NADPH–Cytochrome P-450 Reductase. Detergent-solubilized NADPH–cytochrome P-450 reductase was prepared by the method of Iyanagi et al. (1978). The overall yield of the purified reductase through the preparation was about 25%. Just before use, the reductase was dialyzed against 0.1 M potassium phosphate buffer, pH 7.0, containing 20% glycerol.

Preparation of Cytochrome P-450. A homogeneous preparation of cytochrome P-450 (P-450LM$_2$) was prepared by the method of Imai & Sato (1974) from liver microsomes of phenobarbital-treated rabbits. The P-450 used in this study was gel electrophoretically homogeneous and contained 16.8 nmol of heme protein/mg of protein.

Fluorescence Labeling of NADPH–Cytochrome P-450 Reductase. To each 1 mL of the solution of reductase (20.5 μM as flavin) was added 2.5–32.5 μL of 1 mM ANM (obtained from Wako Chemical Co.) in ethanol, respectively, and the solution was incubated at 20 °C for 2–3 h. When the fluorescence intensity reached a plateau, the reaction mixture was dialyzed against 50 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol at 3 °C for 24 h. The reaction of ANM with reductase was followed as an increase in the absorbance difference at 353 nm (ANM-reacted minus nontreated enzyme) and the fluorescence intensity at 430 nm. The molar ratio of added ANM to the protein was in the range 0.23–3.0. ANM concentration attached to reductase was determined with the extinction coefficient ϵ_{353nm} = 13.18 $mM^{-1}\cdot cm^{-1}$ (Kanaoka et al., 1973). The ANM-labeled reductase at the molar ratio of ANM(bound)/enzyme protein = 1 ± 0.1, in which benzphetamine N-demethylation activity remained unchanged, was used for the reconstitution of the mixed-function oxidase system in phospholipids vesicles. Furthermore, the back-titration of SH groups by a low concentration of pCMB, which has been shown to react with four SH groups of reductase per mol of protein (Nisimoto & Shibata, 1981), indicated that this reductase used for microsomal reconstitution contained ANM stoichiometrically 1:1 per mol of protein.

Reconstitution of Mixed-Function Oxidase System on Phospholipid Vesicles. Reconstitution of P-450 and ANM-labeled NADPH–cytochrome P-450 reductase (ANM/protein ≡ 1.0) in PC/PE/PS = 1:0.5:0.1 (w/w) was performed by the cholate dialysis procedure outlined by Taniguchi et al. (1979) with some modifications. Ten milligrams of DOPC, 5 mg of PE, and 1 mg of PS were freed of the solvent chloroform–benzene under a stream of N_2, further dried under vacuum at 3 °C for 2 h, and suspended in 8 mL of 50 mM potassium phosphate buffer, pH 7.25, containing 0.1 mM EDTA, 0.1 mM DTT, 1% (w/w) sodium cholate, and 20% glycerol, and the mixture was briefly sonicated until clear. P-450 and ANM-reductase were then added, and the resulting lipid–protein–cholate mixture was incubated for 10 h at 3 °C. The dispersion was dialyzed at 3 °C for 48 h against a 500-fold volume of the same buffer as described above except for sodium cholate. The dialysis buffer was changed twice. For reconstitution of ANM-reductase alone in these phospholipids vesicles, the same procedure was used as mentioned above. Ten milligrams of DLPC suspended in 5 mL of 50 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol and 1% sodium cholate was also used for preparation of proteoliposomes. In the present experiments, the fluorescence

measurements were performed for the ANM-reductase incorporated into phospholipid vesicles.

Characterization of Proteoliposomes. Electron micrograph of liposomes containing bound P-450 and ANM-reductase was observed. An aliquot of the liposomes was placed on a specimen mesh coated with a collodion film and then stained with 1% phosphotungstic acid (pH adjusted to 7.1 with KOH). After agarose gel filtration of the reconstituted liposomes (DOPC/PE/PS = 1:0.5:0.1) on Bio-Gel A-150m (Bio-Rad) in 50 mM potassium phosphate buffer, pH 7.25, containing 0.1 mM EDTA, 0.1 mM DTT, and 20% glycerol, the relatively homogeneous proteoliposomes were collected and used in the fluorescent probe study of reductase. In every case 1.2-mL aliquots of liposomal suspension were applied to the column (1 × 35 cm) and eluted at a flow rate of 2 mL/h at 3 °C. Fractions of 0.75 mL were collected and analyzed for their content of phospholipids.

In order to estimate the precise amount of native P-450 incorporated into the vesicles, low-temperature absorption spectra of the reconstituted liposomal fractions at liquid nitrogen were obtained with a Cary spectrophotometer, Model 17. The cell compartment was swept with dried nitrogen gas.

In addition, fluorophotometric assay of total flavin involved in the reconstituted proteoliposomes was performed after the vesicles were treated with 15% trichloroacetic acid (Bessey et al., 1949). After flavin was liberated with trichloroacetic acid (0 °C, 20 min), the precipitates were removed by centrifugation. The supernatant was neutralized at pH 7.2 with 2 N NaOH and then submitted to total flavin determination.

Fluorescence Measurements. Double calcite dichroic polarizers were installed in the excitation and emission paths of a thermostated fluorescence depolarization analyzer (Union Giken FS-501). To approximately equalize the vertically and horizontally polarized components in the excitation beam, a quartz wedge quarter-wave scrambler plate was also placed in the excitation beam between the excitation exit slit and polarizer. The excitation wavelength was selected at 353 nm for ANM by a monochromator. All fluorescence was collected through 400-nm cutoff filters for ANM (Hoya L-40 plus B-440 filter). The relative intensities for the four combinations of vertically (V) and horizontally (H) depolarized excitation and emission beams were recorded in the ratio mode to eliminate source intensity fluctuations. The steady-state emission anisotropies were then calculated as

$$\langle r \rangle = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the relative intensities of the vertical and horizontal fluorescent components when vertically polarized exciting light is used. G is given by I_{HV}/I_{HH}, the ratio of vertical and horizontal fluorescent components with horizontally polarized light and corrects for instrumental bias. Total fluorescence intensity is indicated as

$$I_T = I_{VV} + 2GI_{VH} \quad (2)$$

Temperature dependence of the fluorescence intensity and the emission anisotropy of probes was usually recorded as the temperature was raised unless stated otherwise. Decays of the fluorescence polarization and the fluorescence intensity were measured by a method similar to that described by Kawato et al. (1977, 1980a,b, 1981). The excitation wavelength was the same as that of the steady-state measurements. The filters for fluorescence was a cutoff filter above 390 nm (Fuji SC-37 plus 2 × SC-39 filters). The fluorescence lifetime τ_i and the rotational relaxation time ϕ_j were determined by a curve-fitting procedure. We assumed that the fluorescence intensity $I_T^\delta(t)$ and the emission anisotropy $r^\delta(t)$, responses to a truly impulsive excitation expressed as $\delta(t)$, Dirac's δ function, were expressed as sums of exponential functions:

$$I_T^\delta(t) = \sum_{i=1}^{N} I_i \exp(-t/\tau_i) \quad (3)$$

$$r^\delta(t) = \sum_{i=1}^{M} r_j \exp(-t/\phi_j) \quad (4)$$

Other Methods. Fluorescence spectra were measured with a Shimazu fluorospectrophotometer, Model RF-501, in a sample compartment thermostated at 20 °C. 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). NADPH–cytochrome *c* reductase activity was assayed by the method of Imai (1976). The P-450 concentration was determined from the difference spectrum, reduced CO complex minus reduced, $\Delta\epsilon_{450-490nm}$ = 91 $mM^{-1}\cdot cm^{-1}$ (Omura & Sato, 1964). The concentration of detergent-solubilized NADPH–cytochrome P-450 reductase was estimated from both the absorbance at 456 nm in the absolute spectrum using an extinction coefficient of 21.4 $mM^{-1}\cdot cm^{-1}$ and the molecular weight of the protein (78 000) reported by Iyangi & Mason (1973).

Results

Characterization of ANM-Labeled NADPH–Cytochrome P-450 Reductase. ANM reacts specifically with SH groups in preference to other nucleophilic residues in proteins. The free ANM is nonfluorescent, while the reaction product of ANM with SH groups is strongly fluorescent (Kanaoka et al., 1970). NADPH–cytochrome P-450 reductase has six SH groups per mol of protein in which four are relatively exposed but are not directly involved in the catalytic function, whereas, another two groups are buried and essential for the catalytic activity (Nisimoto & Shibata, 1981). However, the value of 8–10 cysteic acid residues higher than the six free sulfhydryl groups has been detected in other group by titration with 5,5′-dithiobis(2-nitrobenzoate) in the presence of 4 M guanidine (French & Coon, 1979). The specific activity for benzphetamine N-demethylation in the reconstituted phospholipids vesicles containing ANM-reductase and P-450 remained unchanged as the molar ratio of ANM(bound)/enzyme was raised up to 1, beyond which these activities decreased a little (Figure 1C). The absorption and emission spectra bound with reductase are shown in parts A and B of Figure 1, respectively. It is observed that the absorption and corresponding fluorescence traces appear to deviate from one another, especially at the lowest and the highest concentrations of the ANM probe. This deviation seems to be the cause of the displacement of the flavin by incorporation of ANM into the protein. The uncorrected excitation spectrum of ANM bound with reductase had a maximum at 360 nm which was similar to the observed spectrum of ANM attached to cytochrome oxidase (Kawato et al., 1980a,b). The reaction of ANM with reductase was complete within about 3 h at 5 °C since the fluorescence intensity of the probe reached a plateau in this interval. The increased intensity of ANM fluorescence and back-titration of SH groups by a low concentration of pCMB showed that ANM is accessible to only three SH groups exposed probably on a surface of the protein even if the molar ratio of ANM (added)/enzyme was enhanced to 4 or 5. These results are in good agreement with the previous report, indicating that no inhibitory effect was observed with

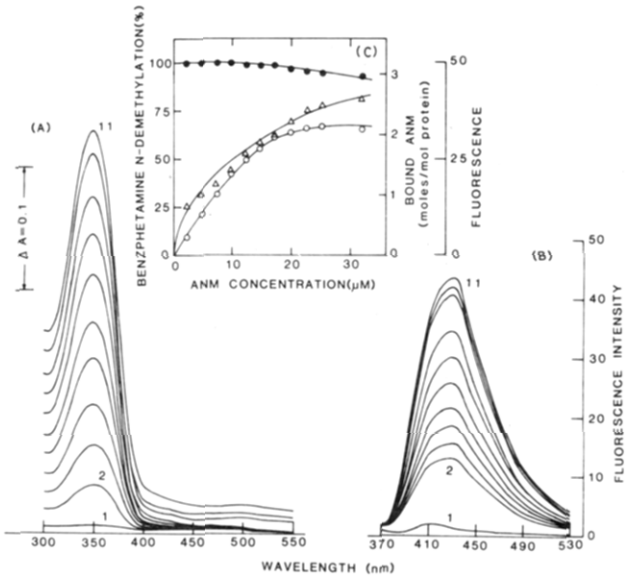

FIGURE 1: Reaction of NADPH–cytochrome P-450 reductase with ANM. One milliliter of the reductase (FMN/FAD = 0.92, 11.1 μM protein) was titrated with various concentrations of ANM (2.5–32.5 μM) for 2–3 h at 20 °C. After modification was completed in this time, nonlabeled free ANM was removed by dialysis against 50 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, and then ANM-labeled minus native difference spectra (A) and the emission spectra of ANM reacted with the reductase (B) were monitored at 20 °C. The experimental curves were not corrected for dilution. (Curve 1) Nontreated enzyme; (curves 2–11) absorption and emission spectra after the reaction of reductase with 2.5, 5.0, 7.5, ..., and 25 μM ANM, respectively. Part C is a plot of the number of bound ANM calculated from the absorbance difference at 353 nm (○) and the increase of fluorescence intensity at 430 nm (△) which occurred as ANM reacted with SH groups of reductase. At the same time, the ANM-modified reductase was used for the assay of benzphetamine N-demethylation activities (●) in the reconstituted mixed-function oxidase system as described under Materials and Methods.

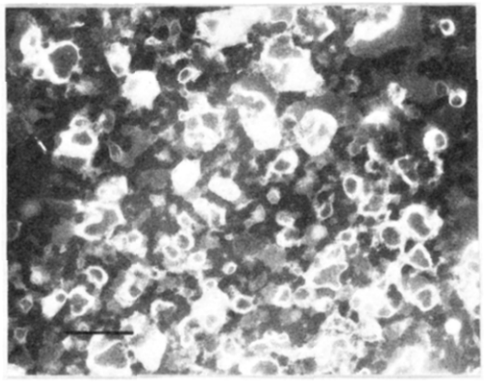

FIGURE 2: Negative-staining electron micrograph of a reconstituted mixed-function oxidase system. The proteoliposomes consisted of ANM-reductase, P-450, and phospholipid mixture (DOPC/PE/PS = 1:0.5:0.1) at a molar ratio of 1/1.3/800. The bar represents 2000 Å.

even high concentrations of *N*-ethylmaleimide (Nisimoto & Shibata, 1981).

Binding of ANM-Labeled Reductase and P-450 to Phospholipid Vesicles. Figure 2 shows a negative-staining electron micrograph of the system reconstituted from P-450, ANM-reductase, and phospholipids (DOPC/PE/PS = 1:0.5:0.1). These proteoliposomal vesicles appear to be approximately 300–1000 Å in diameter. To obtain relatively uniform vesicles, the reconstituted proteoliposomes consisting of P-450 and ANM-reductase were subjected to chromatography on Bio-Gel A-150m columns. Figure 3 illustrates the effectiveness of the

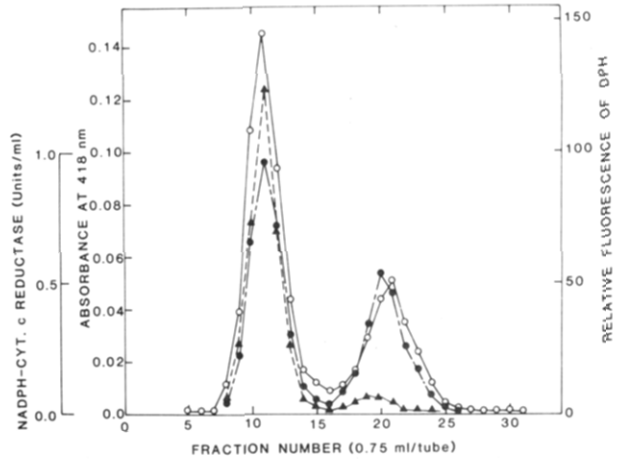

FIGURE 3: Bio-Gel A-150m column chromatography of liposomes containing ANM-reductase and P-450. The roughly reconstituted mixture containing 4.0 μM P-450, 3.7 μM ANM-reductase (ANM/protein ≡ 1), and 3.0 mM phospholipids (DOPC/PE/PS = 1:0.5:0.1) was applied to a 1 × 35 cm Bio-Gel A-150m column equilibrated with 50 mM potassium phosphate buffer, pH 7.25, containing 0.1 mM EDTA, 0.1 mM DTT, and 20% glycerol, and 0.75-mL fractions were collected. The first peak fractions (8–15) containing the two enzymes bound with high concentration of lipids (approximately 3.0 mM) were used for the emission decay assays. The incorporation of DPH into the vesicles was followed as an increase in the fluorescence intensity. The excitation wavelength was selected at 360 nm by a monochromator, and the fluorescence intensity at 440 nm was measured. (○) Absorbance at 418 nm for P-450; (●) NADPH–cytochrome *c* reductase activity, and (▲) the difference of fluorescence intensity between before and after DPH incorporation into liposomes. A concentrated solution of DPH (obtained from Aldrich) in tetrahydrofuran was reacted with each 0.3 mL of fractionated membrane suspension. The reaction mixture was then incubated for 1 h at 30 °C, with occasional mixing. The samples were protected from light during the incubation and immediately subjected to fluorescence measurements.

gel filtration procedure for the separation of proteoliposomes containing the two bound proteins from free proteins. It was shown that 60–70% of the molecules of the two proteins actually formed vesicular complexes with phospholipids, which were rather homogeneous with respect to sedimentation behavior. The first peak fraction containing P-450, ANM-reductase, and the highest phospholipids concentration chromatographed in the void volume was used, in each case, for the fluorescence experiment below. To examine if the P-450 and the ANM-reductase incubated had actually been incorporated into DLPC or DOPC alone vesicles, a reconstituted system consisting of the three components at the same molar ratio as in Figure 3 was subjected to Bio-Gel A-150m chromatography, and the elution behavior of them was monitored by measuring the absorbance increment at 418 nm, NADPH–cytochrome *c* reductase activity, and emission intensity of DPH incorporated into phospholipids. As indicated in Table I, reconstitution of P-450 and ANM-reductase was markedly dependent on the lipid composition of the vesicles. Liposomes prepared from only phosphatidylcholine (DLPC or DOPC) showed a fairly low ability for reconstitution of the heme protein and flavoenzyme, whereas DOPC/PE/PS (1:0.5:0.1) was the most efficient phospholipid mixture for reconstitution. In addition, no significant difference in the incorporation of ANM-reductase into the vesicles (DOPC/PE/PS = 1:0.5:0.1) was observed in the presence and absence of P-450, indicating that ANM-reductase (about 70% of the incubated) is maximally bound to the liposomes between lipid/protein ≡ 5 (w/w) and 10 (w/w) regardless of the presence and absence of P-450. While, as shown in the absorption spectrum of reduced P-450–CO complex at −170 °C (Figure

Table I: Binding of P-450 and ANM-Reductase to Various Phospholipid Liposomes[a]

phospholipids	incubation conditions: P-450/lipids (μmol/mmol)	incubation conditions: reductase/lipids (μmol of protein/mmol)	isolated liposomes: P-450/lipids (μmol/mmol)	isolated liposomes: reductase/lipids (μmol of protein/mmol)
DLPC	1.33	1.23	0.20	0.26
DOPC	1.33	1.23	0.83	0.66
DOPC/PE/PS (10:5:1)	1.38	1.27	1.05	0.87
DOPC/PE/PS (10:5:1)		1.27		0.89

[a] P-450 (4.0 μM heme) and ANM-reductase (3.7 μM protein) were incubated with briefly sonicated phospholipids (2.88–3.0 mM) in 50 mM potassium phosphate buffer, pH 7.25, containing 0.1 mM EDTA, 0.1 mM DTT, 1% (w/w) sodium cholate, and 20% glycerol. The mixture was then dialyzed at 3 °C for 48 h against the same buffer except for 1% (w/w) cholate. Liposomes containing both P-450 and ANM-reductase were then isolated by gel filtration as illustrated in Figure 3. At the same time, the reconstitution and isolation of phospholipid vesicles containing only ANM-reductase were also carried out by the same methods as described above. Analysis of the first peak liposomal fraction in each phospholipid is shown. Thus, the concentration of phospholipid in the isolated liposomes was approximately estimated by a ratio of the fluorescence intensity of DPH measured in the first peak fraction to that in total fractions.

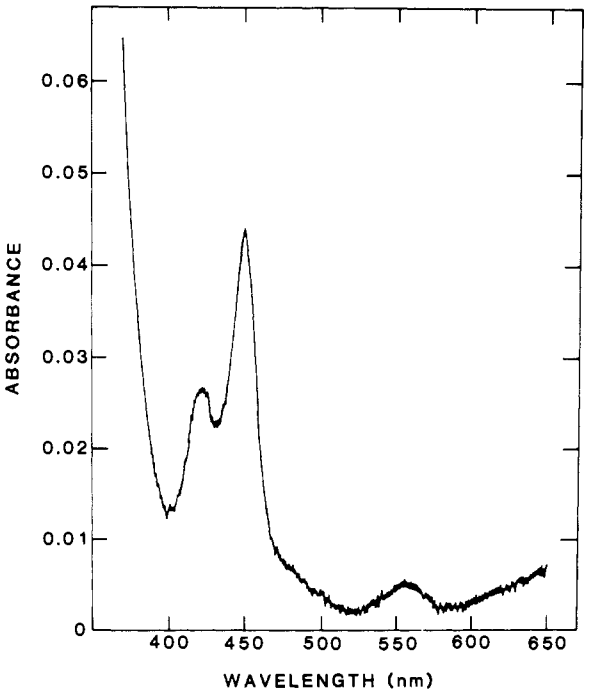

FIGURE 4: Low-temperature absorption spectrum of reconstituted proteoliposomes (DOPC/PE/PS = 1:0.5:0.1). We used the accessory consisting of a specially designed sample cell, thermos bottle, and phototube compartment combination. This special accessory is constructed so that it is easily adapted to perform an absorption measurement in liquid nitrogen. With the accessory properly mounted in both sample and reference cell compartments of a Cary Model 17 spectrophotometer, the beam was focused at the center of the cell position. Dry N_2 was circulated through the cell compartments. Reconstituted vesicles (1.0 μM P-450 and 1.6 μM ANM-reductase) isolated by gel filtration were reduced with 1 mM sodium dithionite dissolved in 10 mM potassium phosphate buffer, pH 8.35, and then reacted with carbon monoxide for 2 min at 0 °C. The sample was allowed to stand in liquid nitrogen, and the spectrum was recorded. The reference cell contained 2 mM phospholipid mixture alone in 50 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol. Light path was 1 mm.

4), the heme protein has been indicated to be effectively incorporated into the phospholipids vesicles, although only a small amount of cytochrome P-420 is observed.

P-450 and Temperature Dependence on the Fluorescence Intensity and Anisotropy of ANM. Direct measurement of the average emission anisotropy was used to establish that ANM-reductase interacts with P-450. In the reconstituted phospholipids vesicles, the presence of P-450 affected the values of I_T and $\langle r \rangle$ of the fluorescent probe bound to reductase. The value of I_T of ANM decreased gradually by increased con-

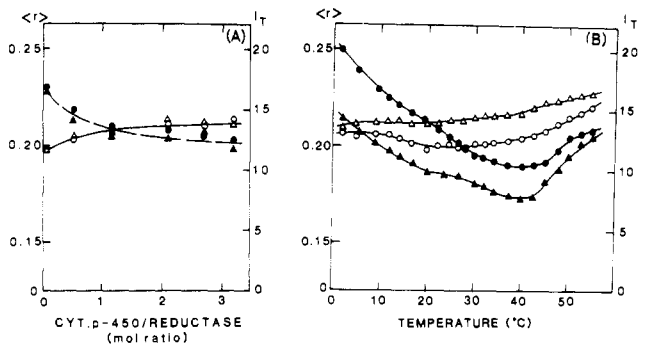

FIGURE 5: P-450 and temperature dependence on the fluorescence intensity I_T and the emission anisotropy $\langle r \rangle$ of ANM-reductase in the reconstituted vesicles. (A) Effect of P-450 on the $\langle r \rangle$ [(○) 1 mM benzphetamine absent; (△) 1 mM benzphetamine present] and I_T [(●) no benzphetamine; (▲) plus 1 mM benzphetamine]. In the estimation of I_T at various P-450 concentrations, the emission intensity of ANM (I_T = 17.5) at [flavin] = 3.5 μM in the proteoliposomes containing only ANM-reductase was regarded as a control. The emission intensity of ANM observed at a slightly varied flavin concentration in each isolated liposomes (P-450/ANM-reductase, 0.48 to 3.10) was converted into the value at [flavin] = 3.5 μM for the correction. Phospholipid (DOPC/PE/PS = 1:0.5:0.1) concentration in each reconstituted vesicles was assumed to be approximately 2 mM, since 90% or more of lipid used for the reconstitution (2 mM) constantly appeared at the first peak fraction as shown in Figure 3. (B) Dependence of temperature on the $\langle r \rangle$ [(○) without P-450; (△) with P-450] and I_T [(●) without P-450; (▲) with P-450]. The reconstituted mixed-function oxidase system contained ANM-reductase, P-450, and phospholipids at a molar ratio of 1/1.3/800. The concentration of ANM-reductase was 1.8 μM as protein (ANM/protein ≡ 1; [flavin] = 3.5 μM).

centrations of P-450 (0.83–5.38 μM heme) in parallel with a negligible increase of $\langle r \rangle$ (Figure 5A). When the molar ratio of P-450 to reductase in the proteoliposomes was approximately 1.4, the observed values (I_T and $\langle r \rangle$) reached almost plateau; however, a precise stoichiometric interaction between the two proteins may be difficult to discuss in only the present study. Since the emission spectrum of ANM-reductase in the reconstituted vesicles partially overlaps the Soret absorption band of the oxidized heme group, the significant depression of the fluorescence emission may be ascribed to excitation energy transfer (Förster, 1965) resulting from the protein–protein association in the membrane of the reconstituted vesicles which is consistent with the results suggested recently by Miwa et al. (1979) and Bösterling & Trudell (1982). Mixing benzphetamine (1 mM) with the reconstituted vesicles gives no significant influence on these values. The steady-state fluorescence intensity and anisotropy of ANM are plotted against temperature in Figure 5B. In the I_T curve of ANM-reductase in the reconstituted mixed-function oxidase

Table II: Effect of P-450 on the Fluorescence Decay of ANM-Labeled Reductase in Phospholipid Vesicles[a]

sample	fluorescence parameters of ANM bound with NADPH–cytochrome P-450 reductase											
	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	$\langle\tau\rangle$ (ns)	r_0	β_1	ϕ_1 (ns)	β_2	ϕ_2 (ns)
(1) ANM-reductase in vesicles	0.57	0.45	0.36	3.16	0.07	8.21	1.96	0.28	0.26	1.91	0.74	161
(2) ANM-reductase/P-450 in vesicles	0.62	0.43	0.33	3.01	0.05	8.07	1.68	0.26	0.26	1.95	0.74	121
(3) (2) + 1 mM benzphetamine	0.60	0.47	0.34	2.99	0.06	7.88	1.77	0.27	0.27	1.48	0.74	105

[a] The total fluorescence $I_T(t)$ is analyzed as a triple exponential decay with three lifetimes, τ_1, τ_2, and τ_3 (solid line in Figure 6). α_1, α_2, and α_3 represent the relative amplitude of each lifetime. $\langle\tau\rangle = \alpha_1\tau_1 + \alpha_2\tau_2 + \alpha_3\tau_3$, average lifetime. The emission anisotropy is expressed as the double-exponential decay fit with two rotational relaxation times (solid line in Figure 6) to the equation $r(t)/r_0 = \beta_1 \exp(-t/\phi_1) + \beta_2 \exp(-t/\phi_2)$. r_0, initial anisotropy. The concentrations of ANM-reductase (ANM/protein ≡ 1), P-450, and phospholipids were the same as those in Figure 6.

system, a small broad depression between 15 and 25 °C and a clear trough around 40 °C were observed. The value of $\langle r\rangle$ showed no marked dependence on temperature, but it began to increase monotonously at about 35 °C and exhibited irreversibility when the temperature was decreased from 55 to 25 °C. Almost similar I_T and $\langle r\rangle$ curves were also seen in the proteoliposomes containing ANM-reductase alone. In view of these results, the increase of the emission anisotropy and intensity above 35 °C seems to be due to a conformational change of ANM-reductase. Although a depression of I_T due probably to the excitation energy transfer from ANM to the heme protein is suggested at each temperature measured, a great change of this value attributed to an amplification of the protein–protein interaction induced by the lipid phase transition is not shown in the range of temperatures measured.

Time-Dependent Emission Anisotropy and Fluorescence Lifetime of ANM. Figure 6 shows decay curves of $I_T(t)$ and $r(t)$ for ANM-reductase in the coreconstituted proteoliposomes containing ANM-reductase and P-450 at 20 °C. Time courses of $r(t)$ in reductase vesicles were biphasic, including a fast phase corresponding to the rapid restricted rotation of ANM bound with reductase, followed by a slower phase which may correspond to the rotation of reductase. All $I_T(t)$ curves of ANM were described as a triple exponential decay in the present solubilized and vesicle systems; however, it is not clear whether these complex decays of $I_T(t)$ are necessarily due to the multiple binding sites of ANM in the enzyme, since the multiexponentiality of these decay kinetics arising from a complex origin is an open question. Fluorescence lifetime and rotational relaxation time of ANM reacted with reductase in the reconstituted liposomes are summarized in Table II. ANM reacted with a small compound had essentially a single lifetime, while ANM bound to enzyme had three lifetime components (Kawato et al., 1981). According to a triple exponential approximation in the total fluorescence decay curve, the longest component of the fluorescence lifetime was about 8 ns. The average lifetime $\langle\tau\rangle$, which is proportional to the quantum yield of the fluorescent probe, is a proper index for comparing the character of the fluorescent probe in different surroundings. Consistent with a significant decrease in the total fluorescence intensity of ANM (Figure 5), an appreciable difference in the magnitude of $\langle\tau\rangle$ for ANM bound with the protein is shown between the absence and presence of P-450 in the reconstituted vesicles. Since the quantum yield of the fluorescence of ANM is sensitive to the polarity around ANM (Kanaoka et al., 1973) and the energy transfer between ANM and heme group (Kawato et al., 1980a,b), the above differences in $\langle r\rangle$ are probably due to the changes in these two factors by the presence of P-450. The marked changes in the fluorescence lifetime and the exponential fraction around 20 °C were not observed after addition of 1 mM benzphetamine to the reconstituted mixed-function

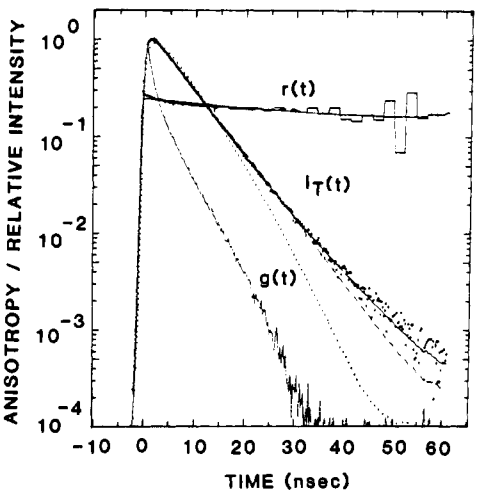

FIGURE 6: Decay curves of the total fluorescence intensity $I_T(t)$ and the emission anisotropy $r(t)$ of ANM bound with reductase in phospholipids vesicles at 20 °C. Proteoliposomes consisting of ANM-reductase (0.93 μM as protein), 1.1 μM P-450, and 0.7 mM phospholipids (DOPC/PE/PS = 1:0.5:0.1) in 50 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM DTT. $g(t)$, the instrumental response function; $I_T(t)$, the total fluorescence intensity (dots, experimental data; dotted, dashed, and solid lines, calculated best-fit curves for single-, double-, and triple-exponential approximations); $r(t)$, fluorescence anisotropy (zigzag lines, experimental; solid lines, calculated best-fit curves for double-exponential approximation).

oxidase system, while random wobbling of ANM (β_1 and ϕ_1) changes little in the presence of P-450, indicating that the fluorescence probe seems to be taking a rapid independent motion in reductase regardless of the existence of the heme protein in the vesicles.

Discussion

Coreconstitution of P-450 and ANM-Reductase into Phospholipid Vesicles. With the availability of purified NADPH–cytochrome P-450 reductase and P-450, it has become possible to reconstitute them into functional units to study metabolism (Lu et al., 1969; Ingelman-Sundberg & Glaumann, 1977; Taniguchi et al., 1979; Bösterling et al., 1979) as well as aspects of the mechanism of protein–protein interactions (French et al., 1980). Evidence was obtained for a binary complex formation (French et al., 1980; Miwa et al., 1979; Bösterling & Trudell, 1982) between the flavoprotein and heme protein. However, some studies were performed with micelle-reconstituted systems that contain only about 20 phospholipid molecules per protein. Therefore, it cannot be excluded that the observed interactions reflect unspecific contacts between these hydrophobic membrane proteins. As shown in Table I, the short phospholipid (DLPC) apparently does not form a membrane bilayer but rather small micelles.

Use of this phospholipid at concentrations below critical micelle concentration together with P-450 and reductase results in catalytically very active P-450–reductase complexes. The earlier study demonstrates that the rate of the P-450-catalyzed mixed-function oxidase reactions in vesicles is strongly dependent on the phospholipid composition (Ingelman-Sundberg et al., 1981; Kawato et al., 1982). It is proposed that the interaction of reductase with P-450 is inefficient in neutral vesicles and dependent upon the negative charge of the liposomal membrane which determines the overall hydroxylation rate in the reconstituted system (Ingelman-Sundberg et al., 1981). This emphasizes that the charge of the membrane is of critical importance for the properties of NADPH–cytochrome P-450 reductase, in particular, for charge-dependent alterations in the functional and conformational properties of the reductase molecule, rather than in P-450 molecule (Ingelman-Sundberg et al., 1981). In reconstituted vesicle systems (lipid to protein ratio is above 5 w/w), PC/PE/PS (1:0.5:0.1 w/w) was the most efficient phospholipid mixture for core-constitution of the two enzymes in the present study, although 100% of the incorporation was not observed even if lipid/protein (w/w) ratio was raised up to above 10. As seen in a previous paper (Ingelman-Sundberg et al., 1980), we also recognized that the presence of PS and/or PE increases the catalytic activities for *p*-nitroanisole O-demethylation and benzphetamine N-demethylation. The acceleration of electron transfer from NADPH–cytochrome P-450 reductase to P-450 by the presence of PS and/or PE was found to be responsible for the above increase in activities. Most recently, Kawato et al. (1982) suggested that PE and/or PS may induce more efficient interactions between the reductase and P-450 resulting in an increase in catalytic activity.

Interaction of P-450 with Reductase in the Membrane of Phospholipid Vesicles. The quenching due probably to resonance energy transfer between ANM bound to reductase and P-450, which is expected from partial overlaps between the emission spectra of ANM and the Soret band of the oxidized heme protein, was observed (Figure 5). As compared with a fairly immobilized motion of ANM in cytochrome oxidase (Kawato et al., 1981), the fluorescent probe showed a considerable motional freedom in the reductase judging from the lower value of the steady-state emission anisotropy (approximately 0.20), suggesting that ANM is exposed on the surface of the protein. If P-450 is closely interacted with ANM-reductase in phospholipid vesicles, one might expect a decrease in the fluorescence intensity induced by the resonance energy transfer. In addition, since the fluorescence intensity appears to be sensitive to the changes in the surroundings of the fluorescent probe, it is also possible that the presence of P-450 leading to a change in the polarity around ANM may induce the change in the intensity. From the temperature-dependent curves in the fluorescence intensity (I_T) of ANM bound to reductase in the vesicles, the thermal quenching which is caused by the collision of the probe with solvent molecules or surrounding amino acid residues is clearly demonstrated (Figure 5B). It is also shown that the peaks in the emission intensity and anisotropy curves observed around 35 °C were irreversible and would be due to a conformational change in reductase (Figure 5B). We could not see that there were marked changes in these quantities which are attributed to a transformation of protein–protein interaction induced by the gel to liquid-crystalline phase transition. In the comparison of $\langle\tau\rangle$ of ANM bound to reductase in the presence and absence of P-450, the decrease of $\langle\tau\rangle$ in the completely reconstituted mixed-function oxidase system (Table II) would reflect the excitation energy transfer between ANM and heme group which is consistent with a significant decrease in the steady-state fluorescence intensity of ANM. The fast decreasing phase faster than 2 ns in $r(t)$ curves of ANM shows the rapid independent wobbling motion of the probe which is not influenced in the presence of P-450. The rotational relaxation time of a hydrophilic head moiety of reductase, which is assumed to be protrudent into the solvent, was longer than 100 ns. As shown by the almost similar rotational relaxation time of this freely mobile group of reductase and reductase associated with P-450, it is suggested that a motion of this hydrophilic group involving ANM-binding site is not greatly affected by the interaction between reductase and P-450 which is probably dependent on translational association of nonpolar segment of the two proteins in the hydrocarbon region of the phospholipid bilayer (Table II). However, we cannot exclude the possibility that P-450 might perturb the flavin environment of the reductase when complex formation between the two proteins was made in the reconstituted phospholipid vesicles. Thus, it may be assumed that the flavins can potentially contribute to I_T loss of ANM as well as the anisotropy changes since they have absorption spectra that overlap the emission of the probe. In fact, the earlier investigation suggested that the possibility of charge–charge interactions plays an important role with respect to the intermolecular interactions of the reductase with P-450 and phospholipid (Black & Coon, 1982; Gum & Strobel, 1979).

Complex formation of reductase with P-450 in the membrane was studied by magnetic CD spectra (Bösterling & Trudell, 1982). The data support a model of specific reversible association reactions in a two-dimensional phospholipid bilayer in which electrostatic interaction are involved because direct electron flow from reductase to P-450 is interrupted by high ionic strength. In addition, the results of a magnetic CD study suggested that a similar electrostatic interaction might also exist between P-450 and cytochrome b_5 in a complete vesicle-reconstituted system (Bösterling & Trudell, 1982). The complex formation between reductase and P-450 has also been indicated by a steady-state kinetic study. Lu & Miwa (1980) investigated steady-state kinetics of substrate hydroxylation of a mixed-function oxidase system consisting of P-450 and reductase in egg phosphatidylcholine vesicles. The change in P-450/reductase ratio affected only the maximal velocity, V_{max}, but the K_m remained unchanged, and maximal activity was achieved with P-450/reductase = 1:1, implying that P-450 forms a 1:1 molecular complex with reductase. Very similar steady-state kinetic data for benzphetamine N-demethylation were obtained by Miwa et al. (1979) in a reconstituted system, supporting a 1:1 molecular complex of P-450 with reductase being essential for catalysis. Furthermore, in the most recent reconstitution experiments, Gut et al. (1982) demonstrated that microsomal P-450 and reductase may form a monomolecular 1:1 complex which catalyzes electron transfer between the two proteins. These recent works are indicating that rabbit P-450 forms a stable 1:1 complex with reductase in membranous reconstituted vesicles as well as nonmebranous reconstituted systems and that various components of the hydroxylation systems are mutually beneficial in favoring binding reactions which lead to the formation of a functional enzyme complex. On the other hand, there are investigations which support the concept of independent lateral mobility of P-450 and reductase in the plane of the membrane. These studies suggest that the interaction between P-450 and reductase is effected by their random collisions caused by lateral mobility in the plane of the membrane. Although it should be noted

that in the present experiment rabbit reductase forms a 1:1 complex with P-450 in a reconstituted membranous system, we show a specific close association of reductase to P-450 in artificial liposomes of a phospholipid composition and protein content resembling that of the natural microsomal membrane, since the I_T of ANM decreased as the molar ratio of P-450/ANM-reductase was raised up to approximately 1, beyond which the I_T stayed almost constant (Figure 5A). The presence of lipid may act to reduce the aggregation of the two proteins, permitting increased interaction between monomeric proteins. Thus, the close association between reductase and P-450 may be facilitated by lipid in this membranous reconstituted system.

We estimate R_0, the critical Förster's transfer distance for the energy transfer with 50% efficiency (Förster, 1965), to be 28 Å for random orientations. The relatively low $\langle r \rangle$ of ANM-reductase and approximately 4-fold symmetry of the heme plane make R_0 relatively insensitive to the orientation factor. If we assume that the changes in ANM fluorescence in the presence of P-450 (Figure 5A) resulted from the formation of a 1:1 complex between P-450 and reductase and that the decrease in I_T was due entirely to the energy transfer, the distance between ANM and heme is calculated to be 35 Å. This value seems to be reasonable to suggest the intermolecular association between the two proteins.

Even though it is well-known that liver microsome contains several different kinds of P-450 isozymes, it is still unclear whether the manner and affinity for the interaction with reductase in vesicles would be varied from different species of P-450. Further studies are required to investigate a possible difference for the molecular interaction between reductase and some different species of P-450 in the reconstituted mixed-function oxidase system.

Acknowledgments

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Registry No. NADPH–cytochrome P-450 reductase, 9039-06-9; cytochrome P-450, 9035-51-2; mixed-function oxidase, 9040-60-2; DLPC, 18285-71-7; DOPC, 10015-85-7; benzphetamine, 156-08-1.

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L-Serine Binds to Arginine-148 of the β_2 Subunit of *Escherichia coli* Tryptophan Synthase†

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ABSTRACT: Inactivation of the β_2 subunit and of the $\alpha_2\beta_2$ complex of tryptophan synthase of *Escherichia coli* by the arginine-specific dicarbonyl reagent phenylglyoxal results from modification of one arginyl residue per β monomer. The substrate L-serine protects the holo β_2 subunit and the holo $\alpha_2\beta_2$ complex from both inactivation and arginine modification but has no effect on the inactivation or modification of the apo forms of the enzyme. This result and the finding that phenylglyoxal competes with L-serine in reactions catalyzed by both the holo β_2 subunit and the holo $\alpha_2\beta_2$ complex indicate that L-serine and phenylglyoxal both bind to the same essential arginyl residue in the holo β_2 subunit. The apo β_2 subunit is protected from phenylglyoxal inactivation much more effectively by phosphopyridoxyl-L-serine than by either pyridoxal phosphate or pyridoxine phosphate, both of which lack the L-serine moiety. The phenylglyoxal-modified apo β_2 subunit binds pyridoxal phosphate and the α subunit but cannot bind L-serine or L-tryptophan. We conclude that the α-carboxyl group of L-serine and not the phosphate of pyridoxal phosphate binds to the essential arginyl residue in the β_2 subunit. The specific arginyl residue in the β_2 subunit which is protected by L-serine from modification by phenyl[2-^{14}C]glyoxal has been identified as arginine-148 by isolating a labeled cyanogen bromide fragment (residues 135–149) and by digesting this fragment with pepsin to yield the labeled dipeptide arginine-methionine (residues 148–149). The primary sequence near arginine-148 contains three other basic residues (lysine-137, arginine-141, and arginine-150) which may facilitate anion binding and increase the reactivity of arginine-148. The conservation of the arginine residues 141, 148, and 150 in the sequences of tryptophan synthase from *E. coli*, *Salmonella typhimurium*, and yeast supports a functional role for these three residues in anion binding. The location and role of the active-site arginyl residues in the β_2 subunit and in two other enzymes which contain pyridoxal phosphate, aspartate aminotransferase and glycogen phosphorylase, are compared.

Studies in our laboratory are aimed at identifying functional residues in the active sites of the α and β_2 subunits of tryptophan synthase of *Escherichia coli* (EC 4.1.2.20) in order to understand the mechanism of action of the separate subunits and of the $\alpha_2\beta_2$ complex. [For reviews of tryptophan synthase, see Yanofsky & Crawford (1972) and Miles (1979).] The α subunit catalyzes the conversion of indole-3-glycerol phosphate to indole and D-glyceraldehyde 3-phosphate (reaction 1); this activity is greatly stimulated by the β_2 subunit. The β_2 subunit catalyzes several pyridoxal phosphate dependent reactions, including the synthesis of L-tryptophan from indole and L-serine (reaction 2) and the deamination of L-serine (reaction 4). The α subunit stimulates reaction 2 and inhibits reaction 4. Reaction 3, the physiologically important reaction, is catalyzed only by the $\alpha_2\beta_2$ complex. Several active-site

indole-3-glycerol phosphate $\rightleftarrows$ indole + D-glyceraldehyde 3-phosphate (1)

indole + L-serine $\rightarrow$ L-tryptophan + H_2O (2)

indole-3-glycerol phosphate + L-serine $\rightarrow$ L-tryptophan + D-glyceraldehyde 3-phosphate + H_2O (3)

L-serine $\rightarrow$ pyruvate + ammonia (4)

residues have previously been identified and located in the primary sequence of the β chain. The lysine which forms a Schiff base with pyridoxal phosphate is Lys-87. A histidine which removes the α-proton of L-serine is either His-82 or His-86 (Higgins et al., 1980; Crawford et al., 1980). Cys-230 is the essential cysteine which is protected from chemical modification by the presence of pyridoxal phosphate (Miles & Higgins, 1980).

In the present study, we have investigated the role of arginyl residues in the β_2 subunit of tryptophan synthase by using chemical modification with the arginine-specific reagent phenylglyoxal (Takahashi, 1968, 1977a,b). Studies with phenylglyoxal and similar reagents containing vicinal carbonyl groups (butanedione and cyclohexanedione) have shown that arginyl residues in many proteins bind anionic ligands, including substrates and cofactors, and are unusually reactive with dicarbonyl reagents (Riordan et al., 1977; Patthy & Thész, 1980). Enzymes which have been selectively modified with dicarbonyl reagents include mitochondrial ATPase (Marcus et al., 1976), carboxypeptidase B (Werber et al.,

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