Pfeuffer, T. (1977) *J. Biol. Chem. 252*, 7224–7234.
Pober, J. S., & Bitensky, M. W. (1979) *Adv. Cyclic Nucleotide Res. 11*, 265–301.
Potter, R. L., & Haley, B. E. (1983) *Methods Enzymol. 91*, 613–633.
Rasenick, M. M., Wheeler, G. L., Bitensky, M. W., Kosack, C. M., Malina, R. L., & Stein, P. J. (1984) *J. Neurochem. 43*, 1447–1454.
Reichert, J., & Hofmann, K. P. (1984) *FEBS Lett. 168*, 121–124.
Shichi, H., & Somers, R. L. (1978) *J. Biol. Chem. 253*, 7040–7046.
Staros, J. V., Bayley, H., Standring, D. N., & Knowles, J. R. (1978) *Biochem. Biophys. Res. Commun. 80*, 568–572.
Swarup, G., & Garbers, D. L. (1983) *Biochemistry 22*, 1102–1106.
Takemoto, D. J., Haley, B. E., Hansen, J., Pinkett, O., & Takemoto, L. J. (1981) *Biochem. Biophys. Res. Commun. 102*, 341–347.
Uchida, S., Wheeler, G. L., Yamazki, A., & Bitensky, M. W. (1981) *J. Cyclic Nucleotide Res. 7*, 95–104.
Van Dop, C., Tsubokawa, M., Bourne, H. R., & Ramachandran, J. (1984a) *J. Biol. Chem. 259*, 696–698.
Van Dop, C., Yamanaka, G., Steinberg, F., Sekura, R. D., Manclark, C. R., Stryer, L., & Bourne, H. R. (1984b) *J. Biol. Chem. 259*, 23–26.
Yee, R., & Liebman, P. A. (1978) *J. Biol. Chem. 253*, 8902–8909.

Effects of Detergent on Substrate Binding and Spin State of Purified Liver Microsomal Cytochrome P-450$_{LM2}$ from Phenobarbital-Treated Rabbits†

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ABSTRACT: Spectral changes accompanying the binding of the nonionic detergent *n*-octyl β-D-glucopyranoside (*n*-octyl glucoside) to cytochrome P-450$_{LM2}$ purified from liver microsomes of phenobarbital-treated rabbits have been compared to changes in catalytic activity obtained in a reconstituted system consisting of various levels of detergent, P-450$_{LM2}$, and NADPH-cytochrome P-450 reductase. In the absence of substrate and reductase, addition of *n*-octyl glucoside to 2–3 mM resulted in a difference spectrum (detergent-bound minus detergent-free cytochrome) characterized by a small maximum at 390 nm and a minimum at 410 nm, suggestive of a slight stabilization of the high-spin ($S = {}^5/_2$) state of the cytochrome. As the detergent concentration was increased to 4–8 mM (corresponding to maximal activity and pentameric or hexameric P-450), a new peak appeared at 427 nm while the minimum remained at 410 nm. Between 10 and 30 mM *n*-octyl glucoside (conditions which produced catalytically inactive and monomeric P-450) the minimum in the difference spectrum shifted to 390 nm and the maximum to 425 nm, characteristic of a shift in spin equilibrium toward low-spin ($S = {}^1/_2$) cytochrome. At low and high detergent concentrations, substrate [*d*-benzphetamine with *n*-octyl glucoside or cyclohexane with the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)] was bound to P-450$_{LM2}$ with formation of high-spin P-450, although the increase in high-spin cytochrome was less at high detergent levels than at low. The affinity of P-450 for substrate decreased by 2–3-fold at high detergent. The decreased substrate affinity is not, however, sufficient to explain the depletion of catalytic activity observed with high detergent. Furthermore, a correlation between spin state and catalytic activity at high detergent levels was not evident. The data suggest the presence of at least two classes of binding sites for amphiphiles: high-affinity site(s) that may correspond to a site essential for catalytic activity and low-affinity site(s) that become accessible as P-450 disaggregates.

Cytochrome P-450$_{LM2}$,[1] the major P-450 isozyme induced in rabbit liver by phenobarbital, catalyzes xenobiotic hydroxylation when reconstituted in solution at a 1:1 molar ratio with NADPH-cytochrome P-450 reductase in the presence of an amphiphile (Coon et al., 1976; Miwa et al., 1979; Guengerich & Holladay, 1979; French et al., 1980; Dean & Gray, 1982; Wagner et al., 1984). The reaction requires NADPH, molecular oxygen, and substrate, and the rates achieved are similar to those observed with intact microsomes (White & Coon, 1980). We recently demonstrated that the nonionic detergent *n*-octyl glucoside (Baron & Thompson, 1975) or the zwitterionic detergent CHAPS (Hjelmeland, 1980) substituted for phospholipid in reconstituted catalytic activity (Dean & Gray, 1982; Wagner et al., 1984). Hydrodynamic studies showed that maximum catalytic activity with either detergent occurred in the absence of formation of a stable complex between P-450 and NADPH-cytochrome P-450 reductase. In the case of *n*-octyl glucoside, maximum rates of *d*-benzphetamine demethylation occurred at 4–8 mM detergent (depending on the preparation used); activity de-

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[1] Abbreviations: P-450$_{LM2}$, the cytochrome P-450 isozyme induced in rabbit liver by phenobarbital; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; *n*-octyl glucoside, *n*-octyl β-D-glucopyranoside; di-12-GPC, dilauroylglyceryl-3-phosphocholine; reductase, NADPH-cytochrome P-450 reductase.

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creased to zero as the *n*-octyl glucoside concentration was increased to 24 mM. P-450$_{LM2}$ was pentameric to hexameric and the reductase monomeric at maximal activity; the cytochrome disaggregated to monomers at the higher detergent concentration. A similar apparent correlation between cytochrome aggregation state and activity was demonstrated with CHAPS when cyclohexane hydroxylation was monitored by the reconstituted system: at 2 mM CHAPS where the system was active, the reductase was dimeric and P-450 was pentameric, while at 20 mM CHAPS, little catalytic activity was exhibited, and the cytochrome was monomeric. Accompanying P-450 disaggregation at higher levels of CHAPS (10–20 mM) was a change in the Soret band of the ferric cytochrome. This CHAPS-induced difference spectrum indicated an altered equilibrium between the high-spin and low-spin states of the porphyrin iron with a slight increase in the low-spin form.

Since changes in spin state have been suggested to modulate the reduction rate of the ferric cytochrome (Sligar et al., 1979; Rein et al., 1979; Backes et al., 1982; Tamburini et al., 1984), it is important to determine if the detergent abolished activity by causing formation of low-spin P-450. Phospholipids such as di-12-GPC have been shown to promote high-spin cytochrome formation, to alter the affinity of P-450$_{LM2}$ for the reductase and substrate, and to promote formation of a stable complex between these two proteins (Coon et al., 1976). In the present paper we studied the ability of two substrates with different amphipaths to elicit a characteristic type I[2] spectral shift of P-450$_{LM2}$ in an attempt to elucidate the nature of the stimulative and inhibitory effects exerted by the two detergents.

Experimental Procedures

Materials. Cytochrome P-450 was purified from the liver microsomes of male New Zealand rabbits weighing approximately 2 kg. The rabbits drank 0.1% aqueous sodium phenobarbital for 4 days prior to killing. Two individual preparations of P-450$_{LM2}$ were used in these experiments. For the first, the purification procedure was similar to that described previously (Coon et al., 1978; Dean & Gray, 1982). For the second, a chromatographic step utilizing carboxymethyl-Sephadex C-50 (Imai et al., 1980) was used subsequent to hydroxylapatite chromatography. For both preparations, the purified cytochrome was washed free of detergent with 0.15 M potassium phosphate, pH 7.5, 20% glycerol, and 0.5 mM EDTA (hereafter referred to as phosphate buffer) while bound to a hydroxylapatite column (Wagner et al., 1984). The specific content of preparation 1 was 12.3 nmol/mg of protein; that of preparation 2 was 16 nmol/mg of protein. In each case the purified P-450$_{LM2}$ was homogeneous as judged by polyacrylamide gel electrophoresis (Laemmli, 1970). The protein was concentrated to 50 or 80 μM in phosphate buffer and stored at −20 °C. CHAPS was recrystallized according to Hjelmeland (1980). Cyclohexane, *d*-benzphetamine, *n*-octyl glucoside, and dilauroylglyceryl-3-phosphocholine were used as received.

Methods. Cytochrome P-450 concentration was estimated by the carbon monoxide difference spectral method (Omura & Sato, 1964) or by the absorbance at 417 nm using an absorptivity to 110 mM^{-1} cm^{-1} (Haugen & Coon, 1976). Protein concentration was estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Optical spectra were recorded from 700 to 300 nm at intervals of 1 nm or from 500 to 300 nm at intervals of 0.5 nm on a Varian-Cary 219 spectrophotometer interfaced to an OLIS Model 3820 data acquisition system (On-Line Instrument Systems, Jefferson, GA). Individual spectra were stored on magnetic diskettes for later processing. Solutions were maintained at 20 ± 0.1 °C with a thermostated water bath and circulator system. The sample curvette contained P-450 which was diluted into the phosphate buffer, and the reference curvette contained phosphate buffer. Detergents, phospholipid, and substrate were added to both curvettes from concentrated stock solutions with a Hamilton microliter syringe. *n*-Octyl glucoside induced difference spectra were generated by computerized subtraction of the spectrum of P-450 obtained initially in the absence of detergent from the spectra recorded after successive additions of the detergent. Difference spectra induced by substrate were generated by subtracting the spectrum recorded in the presence of the amphiphile from that of the cytochrome in the presence of substrate plus amphiphile. Corrections for dilution were made. The substrate-induced ΔA_{max} values were obtained by plotting the reciprocal of the $\Delta A_{387} - \Delta A_{417}$ value vs. the reciprocal of the corresponding substrate concentration. The change in high-spin P-450 was estimated from this extrapolated ΔA_{max} value by using $\Delta\epsilon$ = 110 mM^{-1} cm^{-1} for $\Delta\epsilon_{387} - \Delta\epsilon_{417}$ (Peterson, 1971; Cinti et al., 1979).

Results

n-Octyl Glucoside Induced Difference Spectra of P-450$_{LM2}$. Figure 1 illustrates the complex series of spectral changes observed when P-450$_{LM2}$ was titrated with *n*-octyl glucoside. Difference spectrum 1 in Figure 1A shows that 0.6 mM detergent resulted in the appearance of a small peak at 390 nm and a trough at 420 nm. This change resembles a type I difference spectrum and may indicate a slight increase in high-spin cytochrome. At detergent levels of 1.45 and 2.9 mM (spectra 2 and 3, Figure 1A) there was a general increase in absorbance at 390 nm, a decrease at 410 nm, and the appearance of a new peak at 427 nm. With further additions of detergent (7.9, 13.3, and 23.8 mM; spectra 1, 2 and 3 of Figure 1B), the minimum in the difference spectrum shifted from 410 to 390 nm while the peak at 427 nm increased in magnitude and shifted to 425 nm. These spectral changes suggest that highest detergent levels increased the fraction of low-spin P-450.

In data not shown, the reversibility of the spectral shifts induced by high *n*-octyl glucoside concentration was demonstrated by diluting a P-450 solution which was 24 mM in detergent to a detergent concentration of 1.45 mM. The resulting spectrum (after correction for dilution) was almost identical with one recorded originally at 1.45 mM detergent. The small difference between the two (<6% at 417 nm) resembled the spectrum of the cytochrome, indicating that the discrepancy probably resulted from a slight difference between the calculated and experimental dilution factors.

Titration curves derived from a series of P-450 difference spectra induced by *n*-octyl glucoside are shown in Figure 2. Changes in the millimolar absorptivity differences at two wavelength pairs (390–410 and 425–390 nm) are plotted. These wavelength pairs were chosen as an indicator of low-spin → high-spin conversion and high-spin → low-spin conversion, respectively. The curves show at least two binding phenomena.

[2] The ferric iron of P-450 exists in a thermal equilibrium between a high-spin state characterized by an absorption maximum near 390 nm and a low-spin state characterized by a Soret maximum near 420 nm. In addition, the high-spin state exhibits bands at 500 and 650 nm. Type I substrates tend to stabilize the high-spin state by binding at the active site, while type II substrates coordinate directly to the iron atom and thereby stabilize the low-spin state. A direct correlation between the optical spectra and spin state specific EPR signals has been demonstrated (White & Coon, 1980; Schenkman et al., 1981).

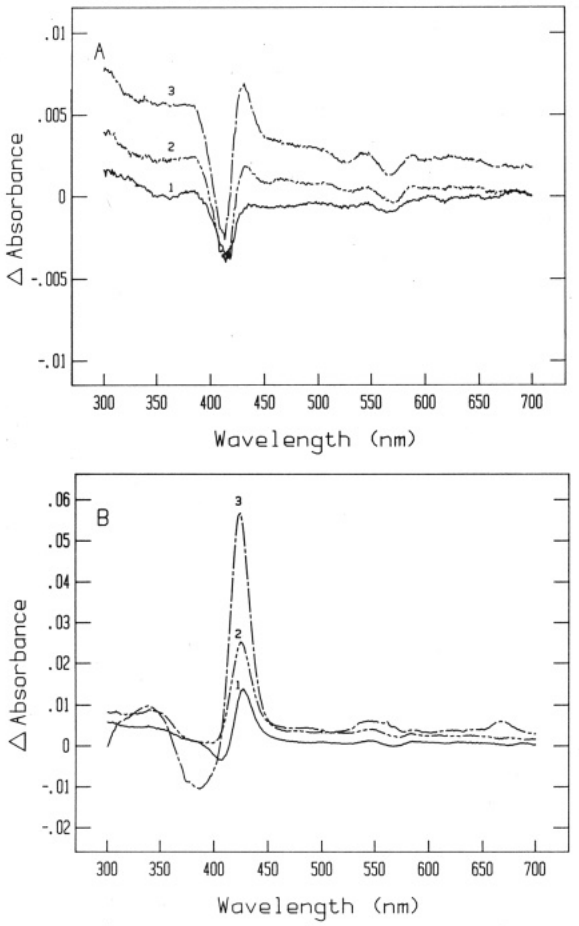

FIGURE 1: Difference spectra of cytochrome P-450$_{LM2}$ induced by various concentrations of *n*-octyl glucoside. Absolute spectra were recorded by computer at the following *n*-octyl glucoside concentrations: (panel A) spectra 1–3, 0.6, 1.45, and 2.9 mM *n*-octyl glucoside, respectively; (panel B) spectra 1–3, 7.9, 13.3, and 23.8 mM *n*-octyl glucoside, respectively. The difference spectra were generated by subtraction as indicated under Experimental Procedures. The conditions were [P-450] = 10 μM (specific content 16.0 nmol/mg), 0.15 M potassium phosphate, pH 7.5, 20% glycerol, and 0.5 mM EDTA, 20 °C.

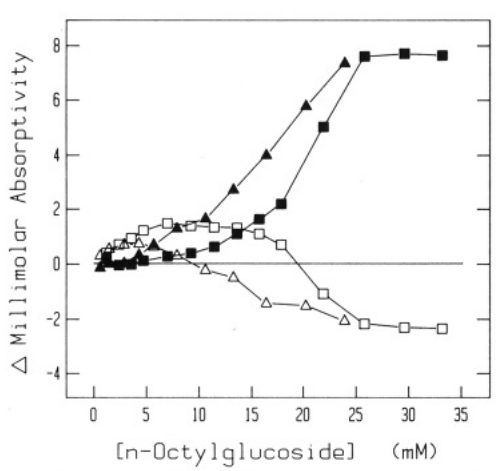

FIGURE 2: *n*-Octyl glucoside binding to cytochrome P-450$_{LM2}$ determined at the wavelength pairs 390–410 (□ and △) and 425–390 nm (■ and ▲). The binding curves were determined for two different P-450 preparations by using spectral titrations similar to those in Figure 1. [P-450] = 10 μM, specific content 16.0 nmol/mg (▲ and △); [P-450] = 9.7 μM, specific content 12.3 nmol/mg (■ and □). Other conditions were the same as in Figure 1.

At lower detergent concentrations a small increase in $\Delta\epsilon_{390-410}$ is evident and appears to approach saturation at about 5–8 mM *n*-octyl glucoside. There is a negligible increase in $\Delta\epsilon_{425-390}$ at these detergent levels. At detergent levels above 10 mM, the absorbance changes at (425–390 nm) are indicative of a cooperative process that approached saturation at detergent concentrations greater than 20–30 mM. The detergent concentration required for saturation varied somewhat depending on the preparation of the cytochrome used. Similar spectral changes were also observed at lower cytochrome concentration (data not shown).

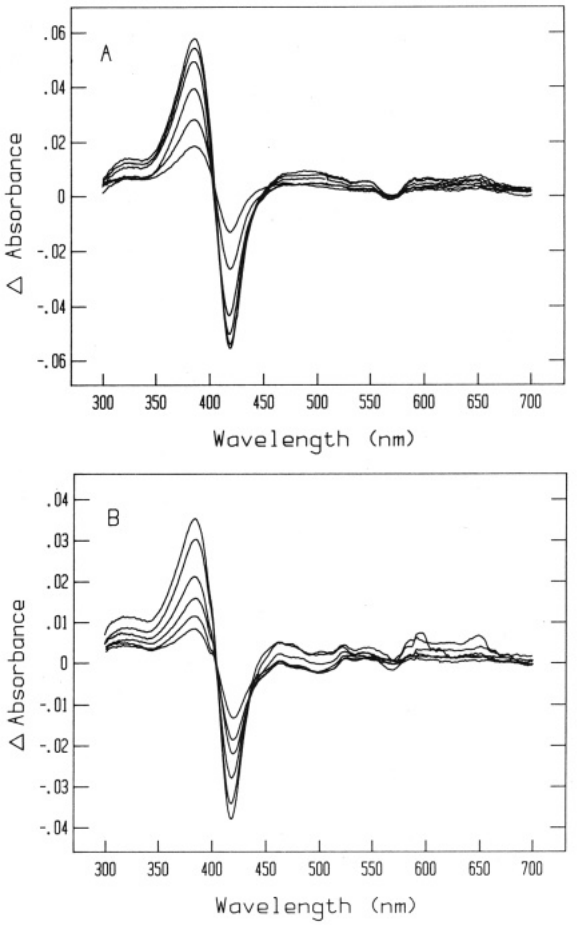

FIGURE 3: Titration of cytochrome P-450$_{LM2}$ (specific content 12.3 nmol/mg) by *d*-benzphetamine in the presence of *n*-octyl glucoside. (Panel A) [P-450] = 5.7 μM, [*n*-octyl glucoside] = 10 mM, and [*d*-benzphetamine] = 0.11, 0.22, 0.43, 0.64, 0.83, and 1.0 mM, respectively; (panel B) [P-450] = 5.4 μM, [*n*-octyl glucoside] = 24 mM, and [*d*-benzphetamine] = 0.21, 0.32, 0.42, 0.62, 1.0, and 1.33 mM, respectively. Other conditions were the same as in Figure 1.

Substrate-Induced Spectral Changes of P-450$_{LM2}$ in the Presence of Different Amphiphiles. The spectral changes in Figure 1B are characteristic of detergent-induced stabilization of low-spin P-450 at the expense of the high-spin form. In order to investigate the effect of detergent-induced changes in spin equilibrium on substrate binding affinity, the ability of cyclohexane and *d*-benzphetamine to elicit a type I difference spectrum was assessed in the presence of CHAPS, *n*-octyl glucoside, and phospholipid. The two substrates were chosen because they have similar high turnover numbers with P-450$_{LM2}$ in the reconstituted system (Coon, 1978; Dean & Gray, 1982; Wagner et al., 1984) and both are type I substrates (Schenkman et al., 1967; van der Hoeven et al., 1974; Hashimoto-Yutsudo et al., 1980). Figure 3 illustrates typical titrations of P-450$_{LM2}$ by using these substrates with different amphiphiles at different concentrations. The substrate and amphiphile in the experiment shown were *d*-benzphetamine and *n*-octyl glucoside at either 10 (Figure 3A) or 24 mM (Figure 3B). The maxima and minima in the difference

Table I: Binding of Type I Substrates to P-450$_{LM2}$ in the Presence of Different Amphiphiles[a]

	[amphiphile] (mM)	K_d (mM)	% change in high-spin P-450 at saturation	substrate-specific rate of NADPH oxidation [nmol of NADPH (nmol of P-450)$^{-1}$ min^{-1}]
n-octyl glucoside +	10	0.5	33.9	20
d-benzphetamine	24	1.0	20.4	0
CHAPS +	2	0.3	29.8	27
cyclohexane	20	0.9	15.9	5
phosphatidylcholine	0.05	0.3	45.2	55
+ cyclohexane				

[a] The apparent dissociation constant (K_d) was derived from the slope of the lines in Figure 4. The percent change in high-spin P-450 was calculated from ($\Delta\epsilon_{387} - \Delta\epsilon_{417}$/110 mM^{-1} cm^{-1}) × 100 (Peterson, 1971); $\Delta\epsilon_{387} - \Delta\epsilon_{417}$ was obtained from the ordinate intercepts of Figure 4. The NADPH oxidation rates are from Dean & Gray (1982) and Wagner et al. (1984).

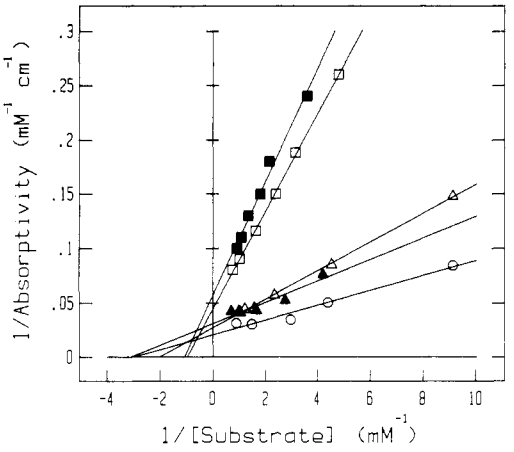

FIGURE 4: Reciprocal plots of spectrophotometric titration of cytochrome P-450$_{LM2}$ by substrate with different amphiphiles. The plots were derived from titrations similar to those shown in Figure 3. Cyclohexane and 20 mM CHAPS (■); *d*-benzphetamine and 24 mM *n*-octyl glucoside (□); *d*-benzphetamine and 10 mM *n*-octyl glucoside (△); cyclohexane and 2 mM CHAPS (▲); cyclohexane and 0.05 mM di-12-GPC (○). The millimolar absorptivities were calculated from the differences $\Delta A_{387} - \Delta A_{417}$. Other conditions were the same as in Figure 1.

spectra are at ~385 and ~417 nm, with isosbestic points at ~407 and ~448 nm; these are typical type I difference spectra. Titrations were also carried out with *d*-benzphetamine in the presence of 0.05 mM di-12-GPC and cyclohexane and in the presence of 2 or 20 mM CHAPS (data not shown). The isosbestic points, maxima and minima, were identical with those of Figure 3. It is apparent from these data that both substrates induce a low-to-high-spin transition even at high detergent concentration where the system was inactive in NADPH-supported hydroxylation.

Reciprocal plots derived from these titrations are displayed in Figure 4 and demonstrate that the affinity of P-450$_{LM2}$ for substrate depends on both the structure and concentration of the amphiphile. A relatively high affinity of cyclohexane or *d*-benzphetamine for P-450$_{LM2}$ occurs with 0.05 mM di-12-GPC, 2 mM CHAPS, or 10 mM *n*-octyl glucoside. Somewhat lower affinity for substrate is evident with higher CHAPS (20 mM) or *n*-octyl glucoside (24 mM) concentration. The extrapolated values of ΔA_{max} obtained at saturation with these substrates also depended on the structure and concentration of the amphiphile, as illustrated by the intercepts at the ordinate of Figure 4.

Turnover numbers, apparent dissociation constants, and the percentage change of high-spin P-450 with different amphiphiles and substrates are summarized in Table I. Although the K_d values increased somewhat in the presence of elevated detergent, the decrease in apparent affinity is not large enough to account for the abolition of catalytic activity at these detergent concentrations. Furthermore, cyclohexane in the presence of high CHAPS concentration and *d*-benzphetamine in the presence of high *n*-octyl glucoside concentration increased the proportion of high-spin cytochrome, although to a lesser degree than at lower detergent concentrations. This behavior is to be expected from consideration of thermodynamically linked equilibria. Finally, there is no apparent correlation between turnover number in these systems and the fraction of high-spin heme iron.

Discussion

In work reported earlier (Wagner et al., 1984) CHAPS was shown by difference spectroscopy to promote spectral shifts in the Soret band of ferric P-450$_{LM2}$. At 2–5 mM, CHAPS caused a small increase in absorbance at 390 nm and a decrease at 420 nm. Similar behavior was consistently observed in the present study with comparable concentrations of *n*-octyl glucoside. Although such changes are characteristic of an increase in high-spin cytochrome, the small magnitude along with the appearance (at high detergent levels) of changes in overlapping regions of the spectrum make the assignment of these changes to perturbations in spin equilibrium somewhat uncertain. Furthermore, the lack of isosbestic points and the multiphasic titration curves obtained on comparison of different wavelength pairs indicates the presence of at least three spectrally distinct species of P-450, two of which may be different low-spin forms. In the previous study, increasing CHAPS to 10–20 mM caused a spectral change similar to a reverse type I difference spectrum, which suggests that the zwitterionic detergent caused an increase in the amount of low-spin P-450. The present results show that similar levels of *n*-octyl glucoside also increase the fraction of low-spin cytochrome; however, the maximum in the difference spectrum was at 425 nm and the minimum was at 390 nm, which differ significantly from those recorded with high CHAPS concentration (427 and 392 nm). These shifts in maxima and minima in the difference spectra suggest that different low-spin forms of P-450 exist in the presence of the different detergents.

The monomeric protein formed in the presence of either 24 mM *n*-octyl glucoside or 20 mM CHAPS was shown not to hydroxylate xenobiotics in a reconstituted system with NADPH-cytochrome P-450 reductase (Dean & Gray, 1982; Wagner et al., 1984). However, the cytochrome in the presence of 20 mM CHAPS retained the ability to hydroxylate substrate in a reaction supported by cumene hydroperoxide (Wagner et al., 1984). P-450 in high detergent concentrations therefore can productively interact with substrate. It has been postulated that NADPH-supported mixed function oxidase depends in mammalian systems on the P-450 spin state (Sligar et al., 1979; Rein et al., 1979; Tamburini et al., 1984). Blanck

and co-workers (1983) reported a correlation between the extent of aerobic reduction of P-450$_{LM2}$ by NADPH–P-450 reductase and the increase in high-spin cytochrome caused by phospholipid. Sligar et al. (1979) showed modulation of the redox potential of partially purified rat liver cytochrome P-450 through thee spin state of the heme iron. On the other hand, Guengerich (1983) found no correlation between activity and spin state. The hydroperoxide mechanism (Nordbloom et al., 1976) should be independent of spin state. Thus, a possible explanation for the lack of activity seen in the reductase-dependent reaction at high detergent might be the inability of the substrate to overcome the detergent-induced stabilization of low-spin P-450 and to promote formation of high-spin P-450. Our results indicate that the lack of activity at high detergent concentration, however, is not a direct result of the inability of substrate to shift the spin equilibrium toward the high-spin state. Neither can it be attributed to failure to bind substrate. Thus, either the monomer itself is for some reason catalytically incompetent or else detergent binding at the low-affinity sites blocks productive interaction between P-450$_{LM2}$ and reductase.

These studies provide direct evidence in addition to our earlier indirect evidence (Dean & Gray, 1982) that detergent binds to P-450 sites of both relatively high as well as lower affinity. The high-affinity site(s) may represent an allosteric effector site, which, when occupied, induces formation of a catalytically active conformation of the protein. There is already considerable evidence that binding of an amphiphile to P-450$_{LM2}$ induces a protein conformational change. Coon et al. (1976) showed that phospholipid increases substrate affinity of P-450. Chiang & Coon (1979) showed by circular dichroism measurements that phospholipid induces a conformational change in P-450, and White & Coon (1982) also suggested that a conformational change may play a role in P-450$_{LM2}$ action. Comparison of the binding kinetics of CO to ferrous P-450$_{LM2}$ in the presence and absence of phospholipid and substrate or detergent plus substrate also demonstrated that simultaneous binding of these ligands resulted in an altered conformation of the cytochrome (Gray, 1983). Finally, adrenal P-450$_{scc}$ possesses a cardiolipin binding site that, when occupied, promotes substrate binding (Lambeth, 1981; Lambeth et al., 1982; Pember et al., 1983).

Müller-Enoch et al. (1984) recently reported that P-450 and reductase form an active complex in the absence of phospholipid if high concentrations of both enzymes are incubated for rather long times. It is conceivable in this case that the role of the amphiphile is tendered by the reductase itself. The hydrophobic membrane-anchoring peptide of reductase (Black & Coon, 1982) might bind at high concentrations to P-450 at the proposed amphipathic effector site.

When compared to di-12-GPC, the ability of the detergents to enhance the catalytic activity of P-450$_{LM2}$ toward type I substrates, despite the inability of these amphiphiles to stabilize high-spin P-450, suggests that the proposed effector site on P-450$_{LM2}$ may operate by a mechanism independent of changes in cytochrome spin state or substrate affinity. Perhaps the conformational change brings the activated oxygen and substrate into juxtaposition for productive hydroxylation (Gray, 1982). Such a mechanism would also provide a role for amphiphaths which are required for hydroxylation by the organic hydroperoxide-supported mechanism.

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Registry No. CHAPS, 75621-03-3; di-12-GPC, 18285-71-7; *n*-octyl glucoside, 29836-26-8; *d*-benzphetamine, 156-08-1; cyclohexane, 110-82-7; reductase, 9039-06-9; cytochrome P-450, 9035-51-2.

REFERENCES

Backes, W. L., Sligar, S. G., & Schenkman, J. B. (1982) *Biochemistry 21*, 1324–1330.

Baron, C., & Thompson, T. E. (1975) *Biochim. Biophys. Acta 382*, 276–285.

Black, S. D., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 5929–5938.

Blanck, J., Rein, H., Sommer, M. Ristau, O., Smettan, G., & Ruckpaul, K. (1983) *Biochem. Pharmacol. 32*, 1683–1688.

Chiang, Y. L., & Coon, M. J. (1979) *Arch. Biochem. Biophys. 195*, 178–187.

Cinti, D. L., Sligar, S. G., Gibson, G. G., & Schenkman, J. B. (1979) *Biochemistry 18*, 36–42.

Coon, M. J. (1978) *Methods Enzymol. 52*, 200–206.

Coon, M. J., Haugen, D. A., Guengerich, F. P., Vermilion, J. L., & Dean, W. L. (1976) in *The Structural Basis of Membrane Function* (Hatefi, Y., & Djavadi-Ohaniance, L., Eds.) pp 409–427, Academic Press, New York.

Coon, M. J., van der Hoeven, T. A., Dahl, S. B., & Haugen, D. A. (1978) *Methods Enzymol. 52*, 109–117.

Dean, W. L., & Gray, R. D. (1982) *J. Biol. Chem. 257*, 14679–14685.

French, J. S., Guengerich, F. P., & Coon, M. J. (1980) *J. Biol. Chem. 255*, 4112–4119.

Gray, R. D. (1982) *J. Biol. Chem. 257*, 1086–1094.

Gray, R. D. (1983) *J. Biol. Chem. 258*, 3764–3768.

Guengerich, F. P. (1983) *Biochemistry 22*, 2811–2820.

Guengerich, F. P., & Holladay, L. A. (1979) *Biochemistry 18*, 5442–5449.

Hashimoto-Yutsudo, C., Imai, Y., & Sato, R. (1980) *J. Biochem. (Tokyo) 88*, 505–516.

Haugen, D. A., & Coon, M. J. (1976) *J. Biol. Chem. 251*, 7929–7939.

Hjelmeland, L. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6368–6370.

Imai, Y., Hashimoto-Yutsudo, H., Satake, H., Girardin, A., & Sato, R. (1980) *J. Biochem. (Tokyo) 88*, 489–503.

Miwa, G. T., West, S. B., Huang, M.-T., & Lu, A. Y. H. (1979) *J. Biol. Chem. 254*, 5695–5700.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lambeth, J. D. (1981) *J. Biol. Chem. 256*, 4757–4762.

Lambeth, J. D., Kitchen, S. E., Farooqui, A. A., Tuckey, R., & Kamin, H. (1982) *J. Biol. Chem. 257*, 1876–1884.

Lowry, O. H., Rosebrough, N. J., Farr, R. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–271.

Müller-Enoch, D., Churchill, P., Fleischer, S., & Guengerich, F. P. (1984) *J. Biol. Chem. 259*, 8174–8182.

Nordbloom, G. D., White, R. E., & Coon, M. J. (1976) *Arch. Biochem. Biophys. 175*, 524–534.

Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370–2378.

Pember, S. O., Powell, G. L., & Lambeth, D. L. (1983) *J. Biol. Chem. 258*, 3198–3206.

Peterson, J. A. (1971) *Arch. Biochem. Biophys. 144*, 678–693.

Rein, H., Ristau, O., Misselitz, R., Buder, E., & Ruckpaul, K. (1979) *Acta Biol. Med. Ger. 38*, 187–200.

Schenkman, J. B., Remmer, H., & Estabrook, R. W. (1967) *Mol. Pharmacol. 3*, 113–123.

Sligar, S. G., Cinti, D. L., Gibson, G. G., & Schenkman, J. B. (1979) *Biochem. Biophys. Res. Commun. 90*, 925–932.

Tamburini, P. P., Gibson, G. G., Backes, W. L., Sligar, S. G., & Schenkman, J. B. (1984) *Biochemistry 23*, 4526–4533.

van der Hoeven, T. A., Haugen, D. A., & Coon, M. J. (1974) *Biochem. Biophys. Res. Commun. 60*, 569–575.
Wagner, S. L., Dean, W. L., & Gray, R. D. (1984) *J. Biol. Chem. 259*, 2390–2395.
White, R. E., & Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315–356.
White, R. E., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 3073–3083.

Transient Kinetics of Adenosine 5′-Triphosphate Hydrolysis by Covalently Cross-Linked Actomyosin Complex in Water and 40% Ethylene Glycol by the Rapid Flow Quench Method†

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ABSTRACT: The initial steps of the ATPase of covalently cross-linked actomyosin subfragment 1 (acto-SF-1) were studied by the rapid flow quench method, and the results obtained were compared with those with reversible (i.e., non-cross-linked) acto-SF-1 and SF-1 under identical conditions. Cross-linked acto-SF-1 plus [γ-^{32}P]ATP reaction mixture milliseconds old was quenched either in a large excess of unlabeled ATP (ATP chase) or in acid (P_i burst). The conditions were pH 8 and 15 °C at 5 mM or 0.15 M KCl and with or without 40% ethylene glycol. In 40% ethylene glycol (5 mM KCl), as with SF-1 and reversible acto-SF-1, the ATP chase was used to titrate active sites and to study the kinetics of ATP binding. Unlike those with SF-1 or reversible acto-SF-1, saturation kinetics were not obtained. The second-order rate constant for ATP binding was 3.1×10^6 M^{-1} s^{-1} for cross-linked acto-SF-1, 1.8×10^6 M^{-1} s^{-1} for reversible acto-SF-1, and 2×10^6 M^{-1} s^{-1} for SF-1. In P_i burst experiments, a transient phase could not be discerned. Because of a high k_{cat}, cross-linked acto-SF-1 was difficult to study in aqueous solution, but at 5 mM KCl, the ATP chase and P_i burst curves were similar to those obtained in 40% ethylene glycol. At 0.15 M KCl the ATP chase curve was difficult to interpret (small amplitude), and there was a small P_i burst. The data obtained in 40% ethylene glycol (5 mM KCl) were adjusted to an abbreviated form of the Bagshaw–Trentham scheme for myosin:

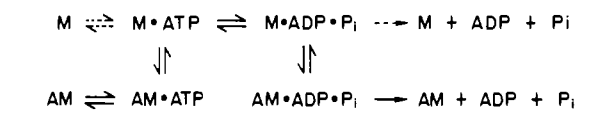
$$AM + ATP \overset{K_1}{\leftrightarrow} AM{\cdot}ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} AM^*{\cdot}ATP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} AM^{**}{\cdot}ADP{\cdot}P_i \xrightarrow{k_4} AM + ADP + P_i$$

The best fit was obtained with $K_1k_2 = 3.1 \times 10^6$ M^{-1} s^{-1} ($K_1 < 1.06 \times 10^4$ M^{-1}, $k_2 > 200$ s^{-1}), $k_3 = k_{-3} = 4$ s^{-1}, and $k_4 = 20$ s^{-1}. With SF-1, $K_1 = 1.25 \times 10^5$ M^{-1}, $k_2 = 16$ s^{-1}, $k_3 = k_{-3} = 8$ s^{-1}, and $k_4 = 0.12$ s^{-1} under the same conditions. Thus, it appears that whereas the kinetics of the ATP binding and the release of products for cross-linked acto-SF-1 and SF-1 are very different, the kinetics of their chemical steps are similar.

Muscle contraction depends on the cyclic interaction of actin and myosin, the energy for which is supplied by the hydrolysis of ATP[1] by the myosin heads. It is thought that the contraction process is modulated by the various intermediates on the ATPase pathway. Therefore, for a full understanding of muscle contraction this pathway must be elucidated.

A reaction pathway for actomyosin ATPase was first proposed by Lymn & Taylor (1971). A key feature of this is that actin dissociates before hydrolysis occurs (Scheme I).

Scheme I[a]



[a] Actomyosin ATPase (dissociative pathway) is indicated by solid arrows. Myosin ATPase is given by the top line. M is myosin and A is actin.

More recent work on the two pathways has lead to an elaboration of the Lymn & Taylor scheme [for reviews, see Trentham et al. (1976), Taylor (1979), Adelstein & Eisenberg (1980), and Sleep & Smith (1981)]. In particular, it is thought that a key step on the myosin pathway is a confor-

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMPPNP, 5′-adenylyl imidodiphosphate; P_i, inorganic orthophosphate; SF-1, myosin subfragment 1; Tris, tris(hydroxymethyl)aminomethane.

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