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BINDING OF TRITON X-100 TO PURIFIED CYTOCHROME P-450scc AND ENHANCEMENT OF THE CHOLESTEROL SIDE CHAIN CLEAVAGE ACTIVITY

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SUMMARY The cholesterol side chain cleavage activity of highly purified adrenal cytochrome P-450scc was enhanced 6-fold by the addition of Triton X-100 in the assay solution in final concentrations of 0.03 to 0.05%, while the same detergent was much less effective in the higher concentrations and Tween 80 was not stimulative to the enzyme in various concentrations. It was shown by gel-filtration chromatography of the P-450 with 0.05 % Triton X-100 that the detergent was bound to the P-450 in an amount greater than 0.5 mg per mg of protein. By the addition of the detergent, 415-nm light absorption of the P-450 was intensified and the isoelectric point was shifted to the alkaline side. Furthermore, the P-450 showed a sedimentation coefficient of 5.1 S in the presence of 0.05 % Triton X-100, whereas it showed a sedimentation coefficient of 8.2S in the absence of the detergent. These results suggest that the observed enhancement of the enzyme activity is largely due to the direct effect of the detergent to the P-450 molecule itself. During these experiments, it was also noted that the P-450 was not resolved into more than one species.

INTRODUCTION

The cytochrome P-450 specific for side chain cleavage (P-450scc) has been isolated from bovine adrenocortical mitochondria as various polymers of apparently identical subunits (1). It cleaves the side chain of cholesterol to produce pregnenolone, while 20- or 22-hydroxycholesterol and 20,22-dihydroxycholesterol are the putative intermediates of the reaction (2). In the light of these observations, we have suspected if our enzyme preparation is a complex of three different enzymes, namely, cholesterol 22R-hydroxylase, [22R]22-hydroxycholesterol 20R-hydroxylase, and [20R,22R]20,22-dihydroxycholesterol 20,22-lyase. However, kinetic and electrophoretic studies favored the one-enzyme-three-step hypothesis rather than the three-enzyme-complex hypothesis (3). In the present work, we further attempted to resolve these three enzyme activities

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Abbreviations used in the present paper ; DCPIP = 2,6-dichlorophenol-indophenol.

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by the aid of various neutral detergents. The attempts provided us an opportunity to observe that the P-450 binds a considerable amount of Triton X-100, while its enzymic activity is significantly enhanced by the binding of the detergent. On the other hand, we were unable to observe the separation of the above-mentioned three enzyme activities.

METHODS

Preparation of cytochrome P-450scc. The P-450 was purified from bovine adrenocortical mitochondria by the modification of the reported method (1). The final product contained 8.6 nmol of heme per mg of protein on the basis of carbon monoxide difference spectrum developed in the presence of 10 μM methyl viologen. It was stored in 50 % glycerol at -20° for months and used in aliquots in the present study.
Analytical methods. The concentration of Triton X-100 was determined by the method of Garewal (4). The side chain cleavage activity was assayed by incubating 20 nmol of [4-14C]cholesterol (about 10,000 cpm in 20 μl of dimethylformamide) with the P-450 (0.1 to 0.5 nmol) for 30 min in the presence of 8.5 nmol of adrenodoxin and 32 DCPIP units of adrenodoxin reductase in a final volume of 1 ml of 100 mM potassium phosphate (pH 7.0) containing 1 mM EDTA, 5 mM cysteine and various concentrations of detergents as indicated below. The produced radioactive pregnenolone was determined by a liquid scintillation spectrometer after separation by thin layer chromatography. Adrenodoxin and adrenodoxin reductase were prepared and assayed by described methods (5,6).
Chemicals. Emulgen 911 and 913 were provided by Kao Sekken Ltd., Tokyo. Triton X-100 and other detergents were purchased. All these detergents were used without purification. The radioactive cholesterol was purchased from CEA, France. Ampholine (pH 3.5 - 10) is the product of LKB, Sweden.

RESULTS AND DISCUSSION

Effect of Triton X-100 and other non-ionic detergents of related chemical structure on the cholesterol side chain cleavage activity of purified cytochrome P-450scc.

Takikawa et al. (7) have shown that addition of a detergent such as Tween 20 in the assay solution results in significant increase of pregnenolone-producing activity of P-450scc. We were likewise able to observe the same effect of various detergents with use of the P-450 preparation refined by our method. It was noted in our experiments, however, that Tween 80 displayed no stimulative effect on the enzyme reaction (Table 1 and Fig. 1). It was noted, furthermore, that there was an optimal concentration of Triton X-100 for the enhancement of the enzyme activity (Fig. 1). The critical micelle concentration (CMC) of Triton X-100 is 0.016 % (8) and does not reconcile with the concentration of the detergent optimal for the enzyme activity. It seems that the hydro-

TABLE I

Effect of Various Non-ionic Detergents on the Enzymic Activity of Purified Adrenal Cytochrome P-450scc

Detergents	HLB-value	Pregnenolone produced
Control (without detergent)	----	2.0 ± 0.2 nmol*
Triton X-100 (POE(9-10)p-t-octylphenyl ether)**	13.5	7.5 ± 0.7
Emulgen 911 (POE(11)p-nonylphenyl ether)	13.7	7.0 ± 0.6
Emulgen 913 (POE(13)p-nonylphenyl ether)	14.5	5.6 ± 0.9
Tween 20 (POE(20)sorbitan monolaurate)	16.7	6.3 ± 1.0
Tween 80 (POE(20)sorbitan monooleate)	16	1.1 ± 0.4
Brij 56 (POE(10) cetyl ether)	12.9	3.6 ± 0.8
Brij 76 (POE(10) stearyl ether)	12.4	2.7 ± 0.7
Brij 96 (POE(10) oleyl ether)	12.4	4.1 ± 1.0

* The figures represent the amount of pregnenolone (mean of 6 incubations ± S.E.) produced by the incubation of 20 nmol of cholesterol for 30 min (see Methods for other conditions). The detergents were added in the final concentration of 0.1 %.
** POE = polyoxyethylene ; the numbers in the parentheses mean the length of the POE chain.

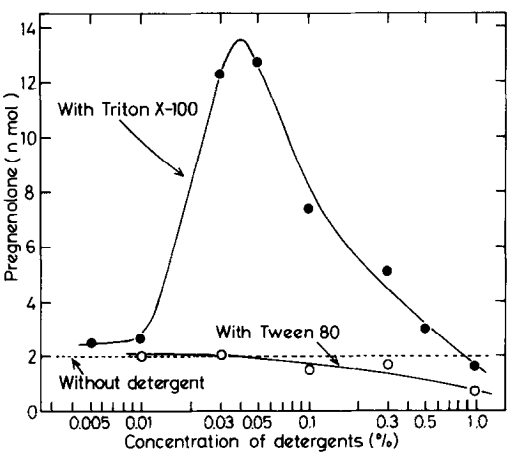

Fig. 1. Effect of the concentration of Triton X-100 and Tween 80 on the cholesterol side chain cleavage activity of cytochrome P-450scc.
The P-450 (0.46 nmol) was preincubated for 10 min at room temperature and for 2 min at 37° in the solution containing the detergent and other components (see Methods), and then the enzyme reaction was started by the addition of NADPH.

phile-lipophile balance (HLB) value of the detergents is also not directly related to the stimulative action of these detergents (Table 1).

It has been known that the cholesterol side chain cleavage reaction shows biphasic kinetics probably because of slow access of substrate to the enzyme (9). When 0.05 %

Triton X-100 was included in the solution, the cleavage reaction proceeded with time in an exponential manner regardless of the length of equilibrium time (data not shown). It seems that the detergent enhances the enzyme reaction by facilitating the access of substrate to the enzyme. Cholesterol has a maximum solubility of 4.7 μM in water, while its CMC is as low as 25 to 40 nM (10). Because cholesterol was used in the present experiment in a concentration 4.3 times as high as its maximum solubility, the stimulative effect of the detergent must be partly due to its effect of increasing solubility of substrate in the solution. Nevertheless, the effect of the detergent can not be solely attributable to its effect on the solubility of substrate, since a detergent is not always stimulative to the enzyme as it was demonstrated above.

Subsequently, we studied the effect of a detergent on the action of adrenodoxin reductase. The rate of reduction of cytochrome C by NADPH in the presence of a mixture of adrenodoxin and the reductase was increased by 41 ± 3 % by 0.05 % Triton X-100. Similarly, the rate of reduction of DCPIP by NADPH in the presence of the reductase was increased by 31 ± 8 % by 0.05 % Triton X-100. The direct effect of the detergent on the ancillary electron carriers, however, may only slightly contribute to the observed increase of the rate of pregnenolone production, since the system we employed contains adrenodoxin and the reductase both in great excess. In this regard, it is relevant to refer to the report of Sugiyama et al. (11) that the reduction of the P-450 by dithionite was greatly accelerated in the presence of Triton X-100. It seems that the effect of the detergent is multilateral, partly on the substrate solubility, partly on the ancillary proteins, and probably to a greater extent on the P-450 molecule itself.

Binding of Triton X-100 to the P-450 .

Figure 2 shows the concentration of Triton X-100 in the eluates of gel-filtration chromatography where the P-450 solution was applied to the column which had been equilibrated with a buffer containing 0.05 % Triton X-100. As it is shown in the insert of the figure, the Triton : P-450 ratio reached a maximum value during the travel of the

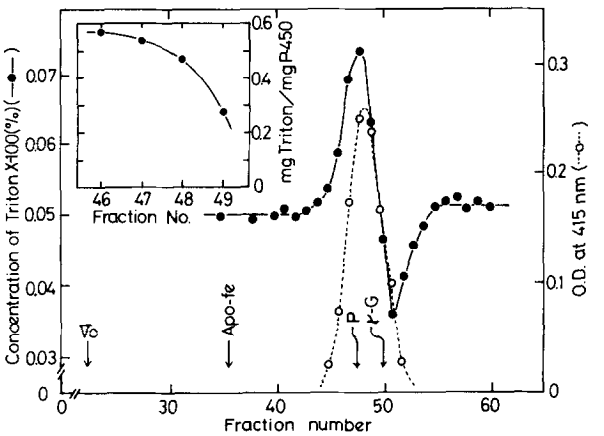

Fig. 2. Gel-filtration chromatography of cytochrome P-450scc in the presence of 0.05 % Triton X-100. One tenth ml of the P-450 solution in 50 % glycerol was mixed with 0.9 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 0.05 % Triton X-100, 0.1 mM EDTA and 0.1 mM dithiothreitol, and applied onto the Sepharose 4B column (1.3 x 58 cm) which had been equilibrated with the same Triton-phosphate as above. The P-450 was eluted with the same buffer at a rate of 10 ml/hr. Each fraction consisted of 1.25 ml.

The inserted figure shows calculated amounts of bound Triton X-100 as a function of the fraction number. The arrow shows the position of the peak of the reference proteins which were chromatographed separately on the same column ; V_o (blue dextran), Apo-fe (apoferritin), γ-G (rabbit gamma-globulin), P (P-450scc in the absence of Triton X-100).

P-450 down the column. It was concluded that the P-450 bound about 0.6 mg of Triton per mg of protein. This is comparable with the value of 1.1 mg of Triton per mg of protein which was estimated by Ingelman-Sundberg et al. (12) for the adrenal another cytochrome P-450 that is specific for 11β-hydroxylation.

It was noted in the present experiment that the detergent caused no change of the rate of elution of the P-450 from the gel-filtration column; V/V_o for the P-450 was constantly 2.2, regardless of whether or not the elution buffer contained Triton X-100. This exclusion volume represents the tetrameric form of the P-450. It is not clear why the P-450 : Triton complex elutes from the column at a rate identical with the rate of elution of the P-450 tetramer.

It has been reported that titration of the P-450 solution with the detergent results in gradual changes of the light absorption spectrum of the P-450 (7,11). We likewise

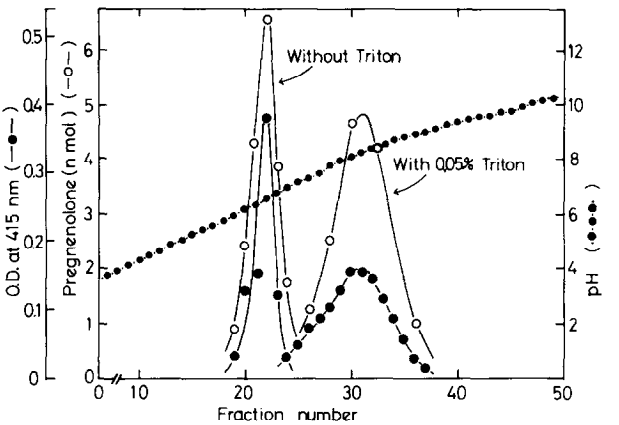

Fig. 3. Isoelectrofocusing of cytochrome P-450scc with or without Triton X-100 (0.05%). A total of 53 nmol of the P-450 was divided into two portions and subjected to isoelectrofocusing in parallel with or without the detergent on 110-ml Ampholine columns (5°, 48 hrs). Two ml fractions were collected and one tenth ml aliquots were subjected to the enzyme assay.

observed that the 415-nm light absorption of the P-450 was increased 1.4 fold when the concentration of Triton X-100 reached 0.05 %. It appears that there is no correlation between the so-called low spin species and the enzyme activity, since no further change of the light absorption was evoked by the increase of the detergent concentration above 0.05 % whereas the enzyme activity was rather diminished by the detergent of higher concentration. Anyhow, the observed change of the light absorption spectrum is indicative of the change of the conformation of the P-450 molecule.

Figure 3 shows the result of isoelectrofocusing of the P-450 with or without Triton X-100. The isoelectric point of the P-450 was shifted to pH 8.2 from the original point at pH 6.5, when 0.05 % Triton X-100 was included in the Ampholine solution. Since Triton X-100 itself is a neutral compound, the observed change of pI of the P-450 must be due to either masking or removal of acidic residues of the P-450. It should be noted in this experiment, furthermore, that there was only one single peak of P-450 among fractions of isoelectrofocusing. Thin layer chromatography of radioactive products of the P-450scc reaction revealed that radioactive hydroxycholesterols were not present in significant amounts in any of the incubation mentioned above.

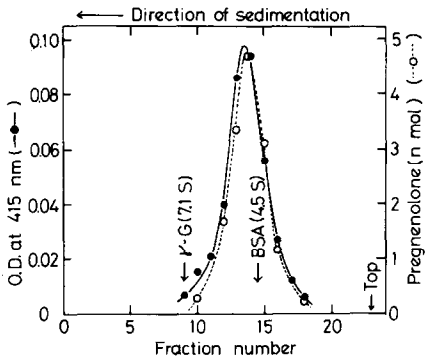

Fig. 4. Velocity sedimentation of the P-450scc (3.7 nmol/tube) on 5-20 % sucrose density gradients (10 ml/tube) containing 0.05 % Triton X-100. Centrifugation was carried out at 4° for 24 hrs. For the assay of the side chain cleavage activity, 150 µl aliquots of each fraction (0.44 ml) were taken. BSA = bovine serum albumin, γ-G = rabbit gamma globulin.

Dissociation of the P-450 by Triton X-100.

We furthermore attempted to resolve the P-450 into the aforementioned three enzyme components by means of the detergent. Thus, the P-450 was subjected to centrifugation on sucrose density gradients containing Triton X-100. As it is shown in Fig. 4, the P-450 sedimented with a sedimentation coefficient of 5.1 S in the presence of 0.05 % Triton X-100. In contrast, the same P-450 showed a sedimentation coefficient of 8.2 S in the absence of the detergent (data not shown). According to the report of Ingelman-Sundberg et al. (12), the P-450(11β) manifests a sedimentation coefficient of 3.1 S on a sucrose gradient containing 0.3 % Triton X-100. It appears that our P-450scc : Triton complex is much larger in size than their P-450(11β) : Triton complex, although the Triton content of the former is seemingly smaller than that of the latter. It may be plausible therefore that the 5.1 S species we observed does not represent monomeric form of the P-450scc. The observation that the presence of Triton X-100 did not alter the rate of elution of the P-450 in gel-filtration chromatography also casts a doubt on the possibility of complete dissociation of the P-450 into its monomer by 0.05 % Triton X-100. More elaborate studies are needed in this context to evaluate the work of Takagi et al. (13) who showed

that the P-450scc is capable of performing the enzymic reaction in a largely polymerized form.

In conclusion, four lines of observations, i.e. (1) binding study by gel-filtration, (2) spectrum change, (3) change of isoelectric point, (4) change of sedimentation coefficient, all suggest that the detergent binds to the P-450 and causes a change of the conformation of the enzyme. The observed enhancement of the enzyme activity may be principally due to this effect of the detergent onto the P-450 molecule itself.

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