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CATALYTIC PROPERTIES OF CYTOCHROME *P*-450scc FROM BOVINE AND PORCINE ADRENOCORTICAL MITOCHONDRIA: EFFECT OF TWEEN20 CONCENTRATION

KAZUHIKO IWAHASHI,[1]* MOTONARI TSUBAKI,[1,4]† AKIRA MIYATAKE,[2] SHIGETOSHI MIURA, KIYOSHI HOSOKAWA[3] and YOSHIYUKI ICHIKAWA[1]

[1]Department of Biochemistry, [2]Research Equipment Center and [3]Department of Neuropsychiatry, Kagawa Medical School, Kagawa 761-07 and [4]Basic Research Laboratory, Himeji Institute of Technology, Himeji, Hyogo 671-22, Japan

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Summary—Cholesterol side-chain cleavage activities of cytochrome *P*-450ssc purified from bovine adrenocortical mitochondria were measured for various substrates, including cholesterol, 20[*S*]-hydroxycholesterol, 22[*R*]-hydroxycholesterol and 20[*R*], 22[*R*]-dihydroxycholesterol, in the reconstituted enzyme system at various Tween20 concentrations. The side-chain cleavage activity for cholesterol showed more than 10-fold enhancement upon addition of 0.1% Tween20, compared with that without the detergent. Addition of Tween20 did not cause any enhancement of the side-chain cleavage activities for 20[*S*]-hydroxycholesterol and 22[*R*]-hydroxycholesterol; rather, it resulted in an inhibition of the activities. The side-chain cleavage activity for 20[*R*],22[*R*]-dihydroxycholesterol showed a very high value even without the detergent. As the stimulatory effect of Tween20 was only specific for cholesterol, Tween20 seemed to enhance the rate of access of cholesterol to cytochrome *P*-450scc. These results are consistent with the suggestion that a transfer of substrate, cholesterol, in mitochondrial inner membrane, to the substrate-binding site of cytochrome *P*-450scc is the rate-limiting step in the cholesterol side-chain cleavage reaction.

INTRODUCTION

The cholesterol side-chain cleavage enzymatic system is common to a variety of steroidogenic tissues (adrenal cortex, ovary, testis and placenta), and its product, pregnenolone, is the common precursor of various steroid hormones. Furthermore, the conversion of cholesterol into pregnenolone [1] is a rate-limiting step in the steroidogenesis, and control of the rate of this reaction is most important for an acute regulation of steroid hormone levels.

This cholesterol side-chain cleavage system consists of three components: an iron–sulfur protein (adrenodoxin); an NADPH-specific flavoprotein containing a single FAD (adrenodoxin reductase); and a heme *b*-containing hemoprotein (cytochrome *P*-450scc). 3 mol each of molecular oxygen and NADPH are required for the conversion of cholesterol to pregnenolone [2]. Hydroxylations occur sequentially at the 22[*R*] and 20[*S*] positions to yield, 22[*R*]-hydroxy and 20[*R*],22[*R*]-dihydroxycholesterol, respectively; both intermediates being tightly bound to the enzyme during the sequential reactions. The third oxidation reaction results in the cleavage of the 20-22 carbon-carbon bond to yield pregnenolone. The side-chain cleavage enzyme shows relatively lower activity for cholesterol, compared with the steroid 11β-hydroxylase (catalyzed by a similar mitochondrial enzyme, cytochrome *P*-45011β, with a turnover rate of 100–120 min-1) even considering the number of steps required for the cholesterol side-chain cleavage reaction, possibly due to the extreme insolubility of its substrate, cholesterol, in water [2]. To overcome this problem, many previous investigators used various detergent and/or artificial phospholipid vesicles to provide access of cholesterol to the cytochrome.

In the present study, we added various concentrations of detergent, Tween20, into the reconstituted enzyme system to investigate its effect more precisely on the side-chain cleavage

*To whom correspondence should be addressed.
†Present address: Basic Research Laboratory, Himeji Institute of Technology.

activity for cholesterol or its hydroxylated intermediates.

EXPERIMENTAL

Bovine cytochrome *P*-450scc, adrenodoxin and NADPH-adrenodoxin reductase were prepared from adrenocortical mitochondria by published procedures [3–6]. Porcine counterparts were purified from adrenal mitochondria using published procedures [3, 4]. Tween20 and cholesterol were obtained from Nacalai Tesque Inc. (Kyoto, Japan). 20[*S*]-hydroxycholesterol and 22[*R*]-hydroxycholesterol were purchased from Sigma (St Louis, MO). 20[*R*],22[*R*]-dihydroxycholesterol, prepared by the method of Morisaki *et al.* [7], was kindly donated by Dr Y. Fujimoto at Tokyo Institute of Technology, Tokyo, Japan. The purity was examined on a gas chromatography–mass spectrometry (GC–MS) to be a single peak.

The enzymatic activity (pregnenolone formation from substrate) was measured using the method of Morisaki *et al.* [8]. 100 μg Each of substrate (cholesterol or its hydroxylated derivatives such as 20[*S*]-hydroxycholesterol,22-[*R*]-hydroxycholesterol, and 20[*R*],22[*R*]-dihydroxycholesterol), dissolved in 50 μl of dimethylformamide, was added in 2 ml of the reconstituted enzyme system at various Tween20 concentrations. After 10 min of preincubation of the reconstituted enzyme system at 37°C, 0.4 ml of NADPH-generating system was added to start the reaction and the reaction was continued for, typically 5 min at 37°C. (The enzymatic activity was linear for at least 10 min for all substrates examined.) The NADPH-generating system consisted of 0.5 ml of 20 mM NADPH, 0.02 ml of isocitrate dehydrogenase (NAD+) (3 IU/ml), 0.15 ml of 0.1 M $MgCl_2$ and 0.05 ml of 0.1 M DL-isocitrate in 20 mM potassium phosphate buffer (pH 7.4) with a final volume of 2.87 ml. The final volume of the reaction mixture was 2.4 ml, which contained: 0.2 μM cytochrome *P*-450scc, 10 μM adrenodoxin, 0.14 μM NADPH-adrenodoxin reductase and various concentrations of Tween20 in 20 mM potassium phosphate buffer (pH 7.4). The reaction was terminated by addition of 5 ml of dichloromethane containing 2.0 μg of [17,21,21,21-^{2}H]pregnenolone prepared by the method of Morisaki *et al.* [8], as the internal standard, followed by vigorous agitation with a vortex mixer for 30 s. After standing for several minutes, the layer of dichloromethane was extracted. The extraction was repeated once more with 5 ml of dichloromethane but without the internal standard. The combined extracts were dried under reduced pressure. After trimethylsilylation, the pregnenolone content was measured by the GC–MS method in the selected ion monitoring mode using the internal standard as described by Morisaki *et al.* [8].

RESULTS

The effects of Tween20 concentration on the side-chain cleavage reaction catalyzed by bovine cytochrome *P*-450scc for cholesterol and its hydroxylated derivatives are shown in Fig. 1 (A, B). The enzymatic activity of cytochrome

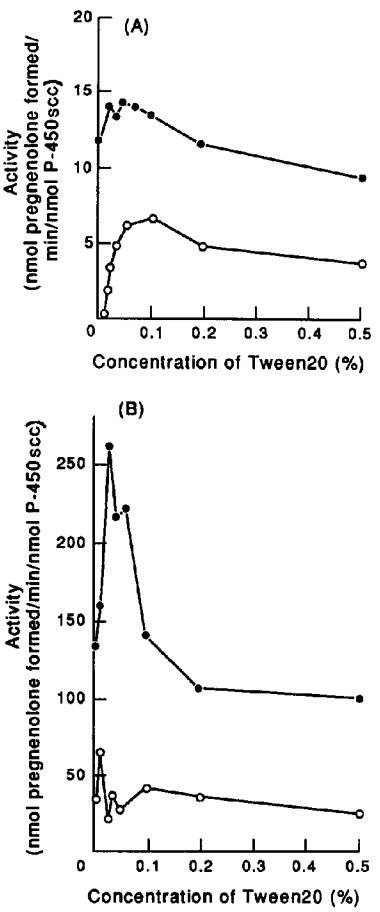

Fig. 1. Enzymatic activities of the reconstituted bovine cytochrome *P*-450scc system at various Tween20 concentrations for (A) cholesterol (○) and 20[*S*]-hydroxycholesterol (●) and (B) 22[*R*]-hydroxycholesterol (○) and 20[*R*],22[*R*]-dihydroxycholesterol (●).

Table 1. Activity ratios of cytochrome *P*-450scc for cholesterol and hydroxycholesterols in the reconstituted system

Substrates	Activity without Tween20[a]	Maximum activity[a]	Ratio
Cholesterol	0.6	7.5	12.5
20[*S*]-Hydroxycholesterol	12	15	1.3
22[*R*]-hydroxycholesterol	26	26	1.0
20[*R*],22[*R*]-Dihydroxycholesterol	135	260	1.9
None	<0.1	<0.1	—

[a]Data are expressed in nmol pregnenolone/min/nmol *P*-450scc as the mean of 3 trials.

P-450scc for cholesterol was only 0.6 nmol of pregnenolone formed/min/nmol *P*-450scc without detergent but showed more than 10-fold enhancement upon addition of 0.1% (v/v) Tween20. However, further addition of Tween20 caused a decrease of the enhancement of the activity as previously reported [9, 10].

The addition of Tween20 did not cause any appreciable enhancement of the activities for 20[*S*]-hydroxycholesterol and 22[*R*]-hydroxycholesterol; it resulted in an inhibition of the activities. The activity for 20[*R*],22[*R*]-dihydroxycholesterol showed a very high value (135 nmol/min/nmol *P*-450scc) even without the detergent and exhibited a maximum (260 nmol/min/nmol *P*-450scc) at 0.02% Tween20. This optimum concentration for 20[*R*],22[*R*]-dihydroxycholesterol is clearly different from that for cholesterol. In Table 1, enzymatic activity ratios of *P*-450scc at the optimum concentration of Tween20 and in the absence of the detergent are tabulated. It is clearly demonstrated that Tween20 showed a considerable activating capability (12.5-fold enhancement) only for cholesterol. For other hydroxylated derivatives its enhancement capability is at most 2-fold.

To obtain a more detailed insight into the enzymatic activities of cytochrome *P*-450scc, we compared the enzymatic activities of bovine and porcine enzymes for various substrates in the presence of 0.1% Tween20. We chose 0.1% Tween20 as the optimum concentration for the reconstituted enzyme system, since it afforded a maximum activity for cholesterol. In Table 2, both of the reconstituted enzyme systems showed remarkable similarities in the activities for each of the substrates examined. It seemed there was no species difference in catalytic properties between these two. The turnover rates (at saturating substrate concentration) were highest for 20[*R*],22[*R*]-dihydroxycholesterol and lowest for cholesterol. When pregnenolone formation was measured using 22[*R*]-hydroxycholesterol or 20[*S*]-hydroxycholesterol as substrate, the turnover rate showed an intermediate value. These observations were in complete accordance with the results obtained from Fig. 1 (A, B).

DISCUSSION

Previously several groups have measured the enzymatic activities of the reconstituted cytochrome *P*-450scc system using nonionic detergents (such as Tween20 or Emulgen913) or artificial phospholipid vesicles.

Takikawa *et al.* [9] reported that the reconstituted bovine cytochrome *P*-450scc system showed a maximum activity in the presence of 0.3% Tween20 for cholesterol as substrate [3]. The V_{max} (measured in the turnover rate) of conversion of cholesterol to pregnenolone under this condition was reported to be 30 min^{-1}37°C, and Kido *et al.* also reported that Tween20 is capable of activating the cholesterol side-chain cleavage activity for cholesterol as substrate, and that the optimal concentration of Tween20 was around 0.21 mM (0.023%) and V_{max} under this condition was 28 min^{-1} [10]. Later, Hanukoglu *et al.* reported that at 0.3% Tween20, the reconstituted enzyme system showed V_{max} between 20–30 min^{-1} for cholesterol as substrate [11]. They also noted that the V_{max} was considerably dependent on the ionic strength of the buffer.

On the other hand, Lambeth *et al.* [1] showed that in an artificial phospholipid vesicle reconstituted cytochrome *P*-450scc system (at 37°C),

Table 2. Enzymatic activities of reconstituted bovine and porcine cytochrome *P*-450scc systems at 0.1% Tween20

System	Substrates[a]			
	Cholesterol	20[*R*],22[*R*]-Dihydroxycholesterol	22[*R*]-Hydroxycholesterol	20[*S*]-Hydroxycholesterol
Bovine system	7.4 ± 0.5	172 ± 28.0	24.3 ± 1.7	14.7 ± 0.5
Porcine system	7.8 ± 1.9	165 ± 18.4	29.0 ± 3.8	19.7 ± 3.4
Blank incubation[b]	<0.1	<0.1	<0.1	<0.1

[a]Data are expressed in nmol pregnenolone/min/nmol *P*-450scc as the mean ± SD for 5 trials. [b]Without the reconstituted enzyme system.

each of the three hydroxylations required for the cholesterol side-chain cleavage reaction occur at approximately the same rate, if the number of hydroxylation steps required for pregnenolone formation from each substrate is taken into account (the calculated V_{max} values were 9.5 min^{-1} for cholesterol, 15 min^{-1} for 22[*R*]-hydroxycholesterol and 31 min^{-1} for 20[*R*],22[*R*]-dihydroxycholesterol) [1]. Tuckey and Stevenson [12] also showed that, at 37°C, the relative turnover rates for 20[*R*],22[*R*]-dihydroxycholesterol (217 min^{-1}),22[*R*]-hydroxycholesterol (102 min^{-1}) and cholesterol (55 min^{-1}) under saturating conditions were almost 1:0.5:0.33 for bovine luteal cytochrome *P*-450scc in phospholipid vesicles, indicating that each of the three hydroxylations occurs at approximately the same rate. It must be noted, however, the turnover rates for each substrate were all 6- to 7-fold higher than those reported by Lambeth *et al.* [1]. Although the turnover rate for cholesterol (at saturating conditions) in our study was considerably lower than those previously reported (except for that reported by Lambeth *et al.* [1], which was very close to our value), it was not due to the partial denaturation of the reconstituted system, since the same system also exhibited a very high turnover rate for 20[*R*],22[*R*]-dihydroxycholesterol, which was close to the value reported by Tuckey and Stevenson [12].

In the present study we have examined the effect of Tween20 for the turnover rates of cytochrome *P*-450scc with the various hydroxycholesterols systematically. Our present data established that, firstly, the turnover rate for 20[*R*],22[*R*]-dihydroxycholesterol was far greater than those for cholesterol, 22[*R*]-hydroxycholesterol or 20[*S*]-hydroxycholesterol. Furthermore, the turnover rate for 22[*R*]-hydroxycholesterol was at least 3-fold higher than that for cholesterol, even comparing them at optimum conditions. Thus, we could not observe any evidence to support that the three hydroxylations required for the side-chain cleavage reaction occur at approximately the same rates, as proposed by Lambeth *et al.* [1] and Tuckey and Stevenson [12]. Secondly, the plausible first intermediate in the side-chain cleavage reaction, 22[*R*]-hydroxycholesterol, has an appreciably higher turnover rate than that of 20[*S*]-hydroxycholesterol. These findings were valid for all the concentrations of Tween20 examined (from 0 to 0.5%).

Lambeth [13] and, later, Kowluru *et al.* [14] have shown that cholesterol side-chain cleavage reactions in phospholipid vesicles containing both cholesterol and cytochrome *P*-450scc are very stimulatory upon addition of specific phospholipids. These stimulatory effects of the phospholipids are, however, not observed for hydroxycholesterol derivatives [1]. This phenomenon may have some relation to the stimulatory effect of Tween20, which is specific only for cholesterol as observed in this study.

The first hydroxylation step to yield 22[*R*]-hydroxycholesterol is apparently the slowest process in the overall reaction of the side-chain cleavage and this process is dependent on the Tween20 concentration. It is known that detergents and phospholipids do not significantly affect the binding of adrenodoxin to the cytochrome, suggesting that the electron transfer rate from adrenodoxin is also independent of the Tween20 concentration [15, 16]. Furthermore, in the cholesterol side-chain cleavage reaction, the supporting electron transfer rate from adrenodoxin is 2–3 s^{-1} when the V_{max} of the side-chain cleavage is 20–30 min^{-1} [2]. The hydroxylation step to yield 22[*R*]-hydroxycholesterol comprises two processes, an access of cholesterol to cytochrome *P*-450scc and 22[*R*]-hydroxylation of cholesterol bound to the cytochrome. It is unlikely that Tween20 enhances the rate of 22[*R*]-hydroxylation reaction without affecting the subsequent hydroxylation steps. Thus, it is inferred that Tween20 facilitates the access of cholesterol to cytochrome *P*-450scc.

Our present results are consistent with the widely-accepted concept that the rate of cholesterol transfer to the cytochrome *P*-450scc active site limits the overall rate of the side-chain cleavage reaction.

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