Vol. 179, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1991 Pages 161-169

HIGH DENSITY LIPOPROTEIN CHOLESTEROL AS A MECHANISTIC PROBE FOR THE SIDE CHAIN CLEAVAGE REACTION

Mikako Takeshima and Takayuki Hara

Section of Biochemistry, Graduate School of Health and Nutrition Sciences, Nakamura Gakuen College, 5-7-1 Befu, Jonan-ku, Fukuoka 814-01, Japan

Received June 17, 1991

Cholesterol side chain cleavage reaction catalyzed by purified cytochrome P-450scc was stimulated 4-5 fold when cholesterol in rat high density lipoprotein was used as a substrate as compared to the case where cholesterol plus 0.1 % Emulgen 911 was used. In the case of the cholesterol-Emulgen system, the V_{max} value of activity was not obtained even when a 20 times molar excess of adrenodoxin over the cytochrome was used. However, in the case of the lipoprotein, a 2-3 times molar excess of adrenodoxin over the cytochrome was enough to obtain the half value of V_{max}. HPLC gel filtration experiments showed that the three enzymes were eluted from the column as a complex regardless of adding the lipoprotein as judged by the activity and the blotting analysis. However, the activity with the lipoprotein was detected significantly earlier than that in the absence. These and other lines of evidence suggest that the lipoprotein vesicles promote a complex formation among the three enzyme components and serve as a probe for emphasizing a significance of a cluster mechanism in the reaction.

Cholesterol side chain cleavage (SCC) reaction is catalyzed by cytochrome P-450scc (P-450scc) and its reducing enzyme components, NADPH-adrenodoxin reductase (AR) and adrenodoxin (AD) in mitochondria of steroidogenic organs (1). This reaction is the rate-limiting step of steroid hormone biosynthesis, and regulated by adrenocorticotropic hormone (2). The reaction involves two sequential hydroxylations at C-22 and C-20, and final cleavage of the carbon-carbon bond between C-20 and C-22 to produce pregnenolone and isocaproaldehyde. Three mol each of

Abbreviations: AD, adrenodoxin; AR, NADPH-adrenodoxin reductase; HDL, high density lipoprotein; LDL, low density lipoprotein; P-450scc, cytochrome P-450 for cholesterol side chain cleavage reaction; SCC, side chain cleavage.

0006-291X/91 $1.50

NADPH and O_2 are required for the overall oxygenation reactions (1).

P-450scc is an integral inner membrane protein of mitochondria, and SCC reaction both in a reconstituted and mitochondrial systems is thus activated by some phospholipids (3,4), peptides (5,6), and proteins (7). However, the mechanism of activation is still unclear because even the reaction mechanism itself is under controvertible situation. Lambeth and colleagues (8,9) have proposed a shuttle mechanism in which AD functions as a mobile electron carrier between AR and P-450scc. Kimura and colleagues (10-12) and Akhrem and colleagues (13,14), however, have proposed a cluster model indicating that a ternary or other higher order complex among the three components is an active form in the reaction.

In this communication, we report that high density lipoprotein (HDL) cholesterol stimulates SCC reaction in a reconstituted system, which is possibly due to the promotion of the complex formation by HDL-vesicles, and serves as a mechanistic probe for SCC reaction.

MATERIALS AND METHODS

Cholesterol oxidase from microorganism was a generous gift from Toyo Jozo (Ohohito, Japan). Other reagents were of reagent grade commercially available. AR, AD, and P-450scc were purified from bovine adrenal cortex and their rabbit antibodies were prepared as described previously (11,12). Purification of HDL and low density lipoprotein (LDL) was carried out from rat serum according to the method of Mitamura (15).

SCC activity was measured essentially as described by Sugano et al. (16). The standard reaction mixture contained 0.8 μM AR, 8 μM AD, 0.4 μM P-450scc, 0.2 mM HDL-cholesterol, 30 mM K-phosphate buffer (pH 7.2), and 0.5 mM NADPH in a total volume of 0.5 ml. When cholesterol was used as a substrate, it was dissolved in propylene glycol as 1 mM, and 0.1 % Emulgen 911 was added to the reaction mixture. After the addition of NADPH, the reaction tubes were incubated at 37° C for 10 min. The reaction was terminated by adding 0.2 ml of methanol, and the tubes were kept in an ice bath for 5 min. To the tubes, 3 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.05 % Tween 20 was added and the tubes were incubated at 37° C for 10 min after addition of 0.2 U of cholesterol oxidase. The reaction was terminated by the addition of 3.5 ml of methanol. Progesterone converted by cholesterol

oxidase from pregnenolone was extracted by 10 ml of petroleum ether, and the extract was evaporated to dryness under an N_2 stream. The dried extract was resolubilized with n-hexane and its aliquot was analyzed by normal-phased HPLC column (Zorbax-Sil, 4.6 mm x 250 mm) equilibrated with a mixture of n-hexane and isopropanol (82:18) and eluted with the same solvent at a flow rate of 1 ml/min. The peak was monitored at 240 nm.

HPLC gel filtration of AR, AD, and P-450scc with or without HDL was performed using PROTEINPAK 300 column (7.5 mm x 300 mm) equilibrated with 10 mM phosphate buffer (pH 7.2) containing 50 mM NaCl and eluted with the same buffer at a flow rate of 0.4 ml/min. The eluate was monitored at 280 nm, and was collected at intervals of 30 sec. Each fractions was used for transfer blotting analysis (17) and NADPH-cytochrome c reduction (11) and SCC activity measurements for the detection of three components. Cholesterol, phospholipid, and protein concentrations were determined by the established methods (18-20). P-450scc content was determined by the method of Omura and Sato (21).

RESULTS AND DISCUSSION

In this study, SCC activity in a reconstituted system was measured using nonradioactive cholesterol as a substrate as described by Sugano et al. (16) with some modifications. We found that the SCC activity was activated 3-4 times by rabbit Anti-P-450scc IgG when cholesterol was used as a substrate (data not shown). Since IgG fraction was prepared from the serum, we measured cholesterol in the IgG fraction (49 mg protein/ml). The concentrations of cholesterol and cholesterol ester were 0.4 mM and 1.6 mM, respectively. This cholesterol was apparently derived from rabbit lipoprotein. We purified HDL and LDL from rat serum to test their stimulation activity. As shown in Table 1, both HDL- and LDL-cholesterol stimulated SCC activity compared with that with cholesterol, but the V_{max} value with HDL was twice larger than that with LDL. The K_m value for cholesterol with HDL was one-third of that with LDL. Since the V_{max} value with HDL is larger than that with LDL, the difference may not be due to the difference in the phospholipid concentrations but that in the phospholipid composition or the apolipoprotein components between them (22). More than 90 % of cholesterol in the HDL-vesicles was metabolized to pregnenolone within 30 min. When we measured

Table 1. SCC activity in a reconstituted system using HDL, LDL, and cholesterol as the substrate

	HDL[a]	LDL[a]	Cholesterol
SCC Activity, V_{max} (nmol/min/nmol P-450scc)	27.7	12.5	7.7
Km for cholesterol (μM)	34	94	535[b]
% of cholesterol metabolized/30 min	>90	45	12

[a]The ratios of cholesterol/phospholipid in HDL and LDL used in this study were 28.8 and 76.6 mol%, respectively. [b]Because of the low solubility of cholesterol, the value may not be exact. Reaction mixtures contained 0.8 μM AR, 8 μM AD, 0.4 μM P-450scc, 0.5 mM NADPH, and various concentrations of cholesterol in 30 mM K-phosphate buffer (pH 7.2). In the case of cholesterol, 0.1 % Emulgen 911 was added.

cholesterol in HDL by the cholesterol oxidase method (18) in the absence of detergent, 91 % of cholesterol was metabolized to 4-cholesten-3-one compared with that in the presence of detergent. These results indicate that most of cholesterol is localized in the outer side of vesicles.

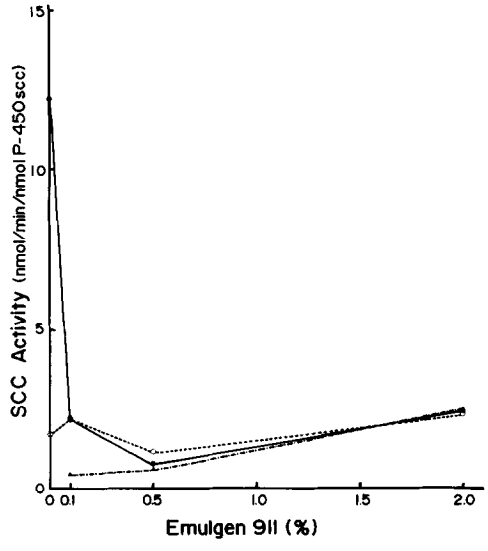


Fig. 1. Effects of detergent concentration on SCC activity using HDL (●), LDL (○), and cholesterol (▲) as the substrate. Various concentrations of Emulgen 911 were added as indicated in the figure and SCC activities were measured. The reaction mixtures were the same as Table 1 except that the concentration of cholesterol was 0.2 mM.

We studied the mechanism of stimulation of SCC activity by HDL-cholesterol. Figure 1 shows the effects of detergent concentration on SCC activity with HDL- or LDL-cholesterol in comparison with that with cholesterol. SCC activity with HDL-cholesterol was inhibited by 94 % in the presence of 0.5 % Emulgen 911, whereas the activities with LDL-cholesterol or cholesterol alone were affected slightly by increasing concentration of Emulgen 911, indicating the necessity of the HDL-vesicle environment for the high activity. We measured SCC activity with HDL-cholesterol using various ratios of AD/P-450scc and compared with that with cholesterol. As shown in Fig. 2, a "lag phase" of activity was observed with cholesterol at AD/P-450scc = 1-2 as reported by Hanukoglu and Jefcoate (23). Evidence for the shuttle mechanism was mainly based on the observation of this "lag phase" (9). However, the "lag phase" disappeared when HDL-cholesterol was used, and the titration curve indicating the stoichiometry of AD/P-450scc = 2-3 was observed. We obtained the same results using liposomal cholesterol consisting of dioleoylphosphatidylcholine (data not shown).

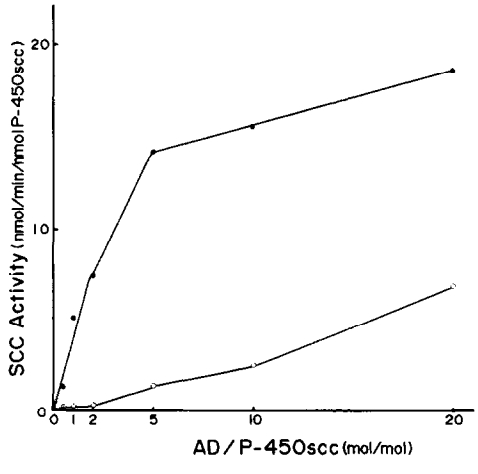
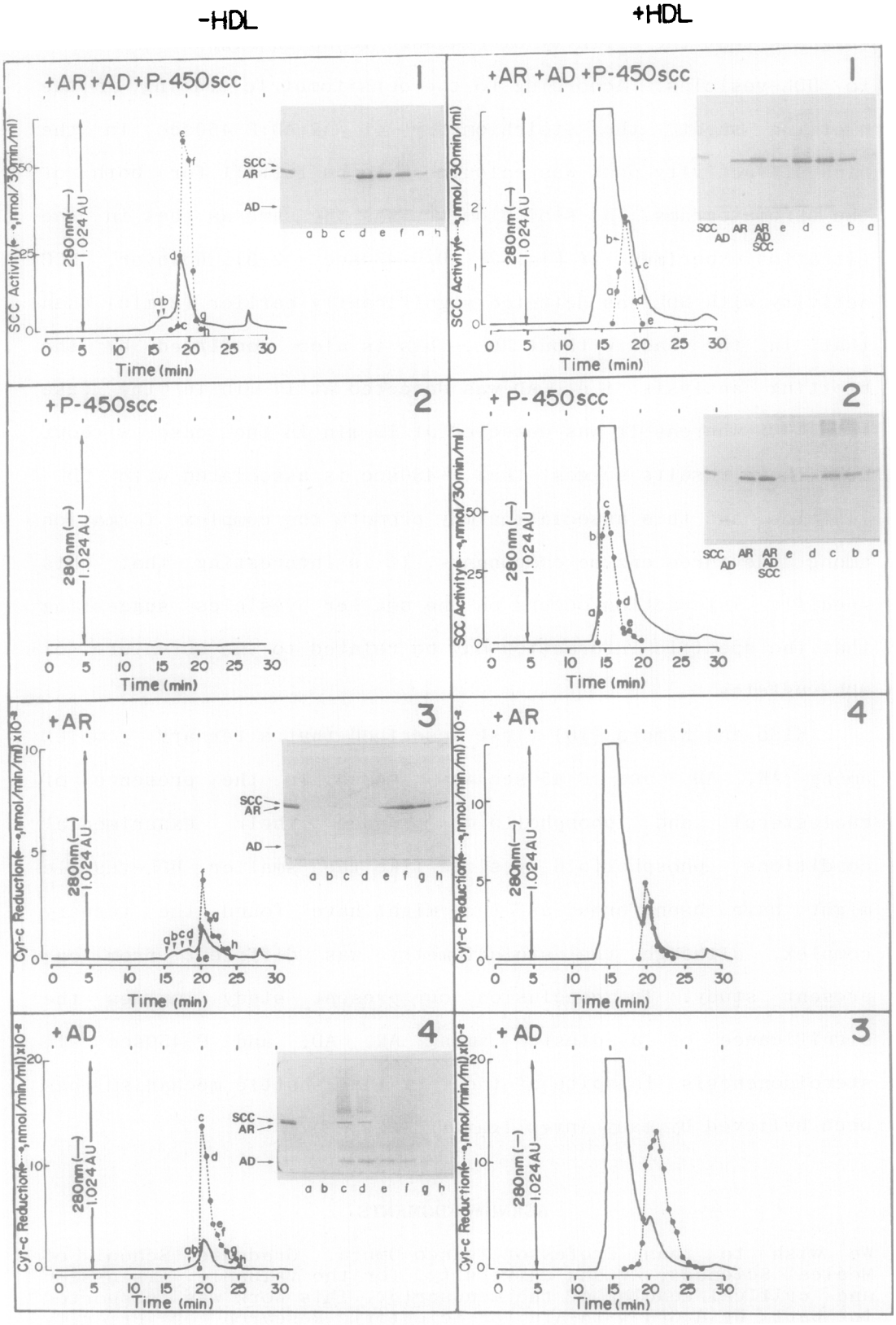


Fig. 2. Titration with AD in SCC activity using HDL (●) and cholesterol (o) as the substrate. Reaction mixtures contained 0.8 μM AR, 0.4 μM P-450scc, 0.2 mM HDL-cholesterol or cholesterol, and various amounts of AD as indicated in the figure. SCC activities were measured as described under "MATERIALS AND METHODS".

Figure 3 shows the elution patterns of AR, AD, and P-450scc when the three enzymes or respective enzyme were mixed with or without HDL-vesicle, and were eluted from PROTEIN PAK 300 column. The numbers in the figures show experimental order using the same column. The insets show the transfer blotting analysis of the three enzymes using purified rabbit antibodies against AR, AD, and P-450scc. P-450scc without HDL was not recovered from the column because of the aggregation in the absence of detergent in the effluent [Experiment 2 (-HDL)]. However, it was eluted together with HDL from the column, and separated into two fractions [Experiment 2 (+HDL)]. The major was associated with the larger vesicles, the minor with the smaller vesicles. This type of association was not seen in AD or AR, because the retention time of AR or AD with and without HDL from the column was exactly the same. Also, P-450scc aggregated in the column was eluted not with AR but with AD (See the blotting analysis). These results indicate that P-450scc is associated with HDL probably due not to the nonspecific protein-protein interaction but to the hydrophobic interactions. The three enzymes both with and without HDL were actually eluted as a complex as judged by the SCC activity or the blotting analysis. Of interest, the major fraction of P-450scc observed in experiment 2 (+HDL) was moved to

---

Fig. 3. HPLC gel filtration of AR, AD, and P-450scc with or without HDL from PROTEINPAK 300 column. The numbers in the figures show the order of experiments using the same column. AR (2 nmol), AD (10 nmol), P-450scc (2 nmol), and HDL (0.9 μmol as the cholesterol) were used for the experiment. The inset shows blotting analysis of the eluate using purified antibodies against AR, AD, and P-450scc. SCC activities of experiment 1 (+HDL), experiment 1 (-HDL), and experiment 2 (+HDL) were measured using 0.1 ml of eluate with nothing, with 0.2 mM cholesterol, and with 0.4 μM AR and 8 μM AD, respectively. NADPH-cytochrome c reductase activities of experiments 3 (-HDL) and 4 (+HDL) and experiments 4 (-HDL) and 3 (+HDL) were measured using 0.1 ml of eluate with 0.05 μM AD and with 0.05 μM AR, respectively. In the blotting analysis, samples equivalent to 0.05 ml of eluate (a-h as indicated as a position in the figures and insets) were applied to an 8 % polyacrylamide slab gel, and then transferred to a nitrocellulose paper. AR, AD, and SCC refer to 0.6 μg of purified AR, AD, and P-450scc, respectively.


-HDL
+HDL
+AR+AD+P-450scc
1
+AR +AD +P-450scc
1
SCC Activity(•—•,nmol/30min/ml)
280nm(—) 1.024AU
Time (min)
SCC
AR
AD
a b c d e f g h
+P-450scc
2
+ P-450scc
2
+AR
3
+AR
4
Cyt-c Reduction(•—•,nmol/min/ml) x10⁻²
+AD
4
+ AD
3

the minor fraction when AR and AD were present. This movement suggests that the affinity of P-450scc to AD is higher than that to HDL-vesicles. According to the densitometric tracing of the blotting sheet, the stoichiometry of AR:AD:P-450scc in the highest activity peak was calculated to be 1:2.4:1 for both of the chromatograms. The stoichiometry was the same as that in the titration experiment of Fig. 2 (AD/P-450scc = 2-3). However, SCC activity with HDL was detected significantly earlier (2 min) than that in the case without HDL. This is also confirmed by the blotting analysis, i.e., AD was detected at 17 min in the case with HDL whereas it was detected at 19 min in the case without HDL. These results suggest that P-450scc is associated with HDL-vesicles, and this association may promote the complex formation among the three enzyme components. It is interesting that this specific interaction occurs on the smaller vesicles suggesting that the specific interaction may be related to the curvature of HDL-vesicles.

Kido and Kimura (10) first described that a ternary complex among AR, AD, and P-450scc was formed in the presence of cholesterol and phospholipid. Under their experimental conditions, phospholipid vesicle like the smaller HDL-vesicle might have been formed and they might have found the ternary complex, although the stoichiometry was different from our present study. In conclusion, our present study implies the significance of a cluster among AR, AD, and P-450scc in steroidogenesis in spite of the fact that shuttle mechanism has been believed by many investigators (8,9,23).

ACKNOWLEDGMENTS

We wish to thank Professor Tsuneo Omura, Graduate School of Medical Sciences, Kyushu University, for the valuable discussion and critical reading of the manuscript. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority

Areas, "Molecular Biology of Cytochrome P-450" (02217216) from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. Takemori, S., Suhara, K. and Katagiri, M. (1978) in: Cytochrome P-450 (Sata, R. and Omura, T. eds) pp.164-184, Kodansha, Tokyo and Academic Press, New York and London.
2. Kimura, T. (1981) Mol. Cell. Biochem. 36, 105-122.
3. Seybert, D. W., Lancaster, J. R., Lambeth, J. D. and Kamin, H. (1979) J. Biol. Chem. 254, 12088-12098.
4. Kowluru, R. A., George, R. and Jefcoate, C. R. (1983) J. Biol. Chem. 258, 8053-8059.
5. Kido, T. and Kimura, T. (1981) J. Biol. Chem. 256, 8561-8568.
6. Pedersen, R. C. and Brownie, A. C. (1987) Science 236, 188-190.
7. Yanagibashi, K., Ohno, Y., Kawamura, M. and Hall, P. F. (1988) Endocrinology 123, 2075-2082.
8. Lambeth, J. D., Seybert, D. W. and Kamin, H. (1979) J. Biol. Chem. 254, 7255-7264.
9. Lambeth, J. D., Seybert, D. W., Lancaster, J. R., Salerno, J. C. and Kamin, H. (1982) Mol. Cell. Biochem. 45, 13-31.
10. Kido, T. and Kimura, T. (1979) J. Biol. Chem. 254, 11806-11815.
11. Hara, T. and Kimura, T. (1989) J. Biochem. 105, 594-600
12. Hara, T. and Kimura, T. (1989) J. Biochem. 105, 601-605
13. Chashchin, V. L., Turko, I. V., Akhrem, A. A. and Usanov, S. A. (1985) Biochim. Biophys. Acta 828, 313-324.
14. Usanov, S. A., Turko, I. V., Chashchin, V. L. and Akhrem, A. A. (1985) Biochim. Biophys. Acta 832, 288-296.
15. Mitamura, T. (1982) J. Biochem. 91, 25-29.
16. Sugano, S., Morishima, N., Ikeda, H. and Horie, S. (1989) Anal. Biochem. 182, 327-333.
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Clonong: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W. and Fu, P. C. (1974) Clin. Chem. 20, 470-475.
19. Ames, B. N. and Dubin, D. T. (1960) J. Biol. Chem. 235, 769-775.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
21. Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2379-2385.
22. Chapman, M. J. (1986) Methods Enzymol. 128, 70-143.
23. Hanukoglu, I. and Jefcoate, C. R. (1980) J. Biol. Chem. 255, 3057-3061.