PARTIAL RESOLUTION OF THE MIXED-FUNCTION OXIDASE INVOLVED IN THE CHOLESTEROL SIDE-CHAIN CLEAVAGE REACTION IN BOVINE ADRENAL MITOCHONDRIA

E.R. Simpson and G.S. Boyd

Department of Biochemistry,
University of Edinburgh Medical School,
Teviot Place,
Edinburgh.

Received August 9, 1967

In recent years, the role of cytochrome P-450 as the site of substrate and or oxygen activation in mixed function oxidases has been clearly established in a number of cases (Estabrook, Cooper and Rosenthal, 1963; Omura, Sato, Cooper, Rosenthal and Estabrook, 1965). Such systems possess the property of light-reversible carbon monoxide inhibition. The partition constant for the carbon monoxide inhibition is about unity, and the photochemical action spectrum for the light-reversal shows a maximum at 450 mμ, similar to the optical absorption spectrum of P-450 (Omura et al., 1965).

We have demonstrated that these criteria of mixed function oxidase activity apply to the cholesterol side-chain cleavage system of adrenal cortex mitochondria, which effects the conversion of cholesterol to pregnenolone. This reaction is an early step in the metabolism of cholesterol to the adrenal steroid hormones, and occurs without detectable formation of intermediates (Simpson and Boyd, 1967a and 1967b). Another key step in the biosynthesis of the adrenal steroid hormones is 11β-hydroxylation. The enzyme system effecting this reaction has been purified from adrenal cortex mitochondria and

shown to consist of three protein components - a flavoprotein, a non-haem iron protein, and a fraction containing P-450. The former two constitute a NADPH - cytochrome P-450 reductase (Omura, Sanders, Estabrook, Cooper and Rosenthal, 1966).

We have purified the cholesterol side-chain cleavage system using molecular sieve and ion-exchange chromatography. The purpose of this communication is to present evidence that this system appears to have much in common with the steroid 11ß-hydroxylase, with regard to the protein components involved.

Methods

Preparation of bovine adrenal cortex mitochondria and enzyme assay using cholesterol-4-C^{14} were as previously described (Simpson and Boyd, 1966.) except that the T.L.C. system used was petroleum ether:diisopropyl ether:acetic acid ::: 30:70:2 (2 runs).

Acetone powder of mitochondria was prepared by pouring the mitochondria, suspended in 0.154M KCl, into 20 volumes of acetone at 20°C., with violent agitation. The suspension was filtered, washed four times each with acetone and diethyl ether (both at -20°C) and then dried under vacuo. The resulting off-white powder contained cholesterol side-chain cleavage activity which could be extracted from the powder by hand homogenisation in 0.1M phosphate buffer pH 7.4 and centrifugation at 105,000 x g for 30 min. The clear supernatant from 15 mg. protein converted about 1 per cent of the cholesterol substrate to pregnenolone in 1 minute in the standard assay.

Non-haem iron was determined after the method of Massey (1957) except that the samples were made 2N with HCl before addition of the trichloracetic acid. Pyridine (1 ml.) was added instead of ammonium citrate, and the iron reagent used was bathophenanthroline sulphonate rather than o-phenanthroline. The optical density was read at

535 mμ. Haem iron was determined after the method of Adler and George (1965) on the protein precipitates from the 'non-haem iron' determination. Again the iron reagent used was bathophenanthroline sulphonate. NADPH-diaphorase activity was measured after the method of Omura et al. (1966).

Results and Discussion

Acetone powder (1800 mg. from about 80 g. adrenal cortex) was extracted with 120 ml. 0.1M phosphate buffer. The extract was con-

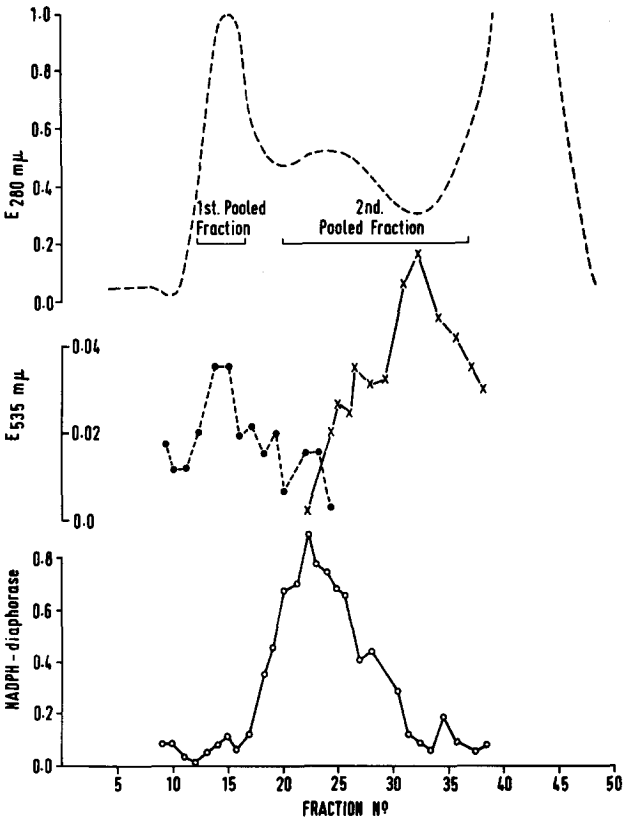

Fig. 1. Chromatography of acetone powder extract on Sephadex G-200

- - - - E_{280}
x——x non-haem iron
● - - ● haem iron
o——o NADPH-diaphorase activity

centrated to 40 ml. with polyethylene glycol (Carbowax, Union Carbide) and subjected to ultrasonication at 20 Kc/sec. for 5 min. at $0^{\circ}C$. This solution was applied to a column of Sephadex G-200 4.5 cm. by 25 cm. previously equilibrated with 0.04M phosphate buffer. Elution was continued with this buffer at $4^{\circ}C$ and 8 ml. fractions collected. Fig. 1 shows the elution pattern.

Fractions were combined as shown, and the pooled fraction containing the haem iron was concentrated, sonicated once more for 5 minutes and re-chromatographed on the G-200 column. The fractions containing the highest protein concentration were pooled. These fractions contained cytochrome P-450 as shown by its reduced carbon monoxide difference spectrum (Omura et al, 1965).

The second pooled fraction from the G-200 column, containing NADPH-diaphorase activity together with the non-haem iron protein was run on to a column of DEAE Sephadex A-25 2.5 cm. by 25 cm. equilibrated with 0.04M phosphate buffer. Elution was effected by means of a sodium chloride gradient up to 0.7 M. Fig. 2 shows the elution pattern. The protein peak containing non-haem iron possessed an absorption spectrum characteristic of adrenal mitochondrial 'adrenodoxin' (Kimura and Suzuki, 1967). Neither the P-450 fraction nor the NADPH-diaphorase nor the non-haem iron protein was completely pure by the criteria of ultracentrifugation and zone electrophoresis in acrylamide gel and cellulose acetate.

These components were then examined for cholesterol side-chain cleavage activity by incubating with cholesterol-4-C^{14} at $37^{\circ}C$ for 15 min. in the presence of a NADPH-generator. Table 1 shows that reconstitution of cholesterol side-chain cleavage activity was achieved only in the presence of the NADPH-diaphorase, plus the non-haem iron protein and the fraction containing P-450. The concentration of each component corresponded to material from 30 mg. original

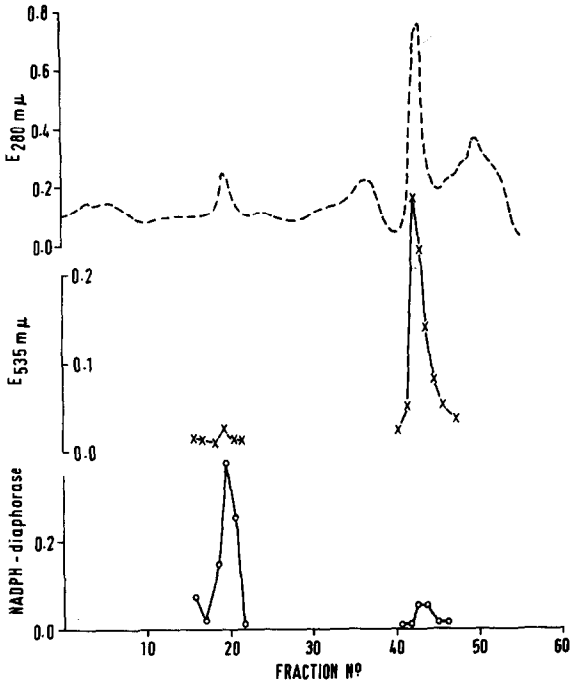

Fig. 2. Re-chromatography of the pooled fraction containing non-haem iron and NADPH-diaphorase activity on DEAE-Sephadex A-25

- - - - E_{280}
x——x non-haem iron
o——o NADPH-diaphorase activity

TABLE 1.

Reconstitution of cholesterol side-chain cleavage activity

Composition of incubation	Rate of conversion to pregnenolone (percentage metabolised in 15 min.)
P-450 plus NADPH - diaphorase	0.8
P-450 plus non-haem iron protein	4.1
NADPH - diaphorase plus non-haem iron protein	3.4
P-450 plus non-haem iron protein plus NADPH - diaphorase	18.5

acetone powder i.e. twice the concentration required to achieve the same conversion in unfractionated material. Halving the concentration of each component completely abolished the activity.

The cholesterol side-chain cleavage system of adrenal cortex mitochondria appears to consist of a fraction containing cytochrome P-450 and an NADPH-cytochrome P-450 reductase composed of a flavoprotein and non-haem iron protein. This enzyme complex appears to be similar to the corresponding enzyme complex of the 11β-hydroxylase of bovine adrenal cortex (Omura et al. 1966), and furthermore has not been differentiated from it, suggesting that both mixed-function oxidase activities may reside in the same complex. The side-chain cleavage reaction may be the rate-limiting step in steroidogenesis in the adrenal cortex, so any of these protein components could be the overall rate-limiting factor. Rigorous purification of the proteins involved will be necessary to settle these questions.

This work was supported by a grant from the Scottish Hospital Endowments Research Fund (Hert 128).

References

Adler, A.D. and George, P., Anal. Biochem., 11, 159 (1965).
Estabrook, R.W., Cooper, D.Y. and Rosenthal, O., Biochem. Z., 338, 741 (1963).
Kimura, T. and Suzuki, K., J.biol. Chem., 242, 485 (1967).
Massey, V., J.biol. Chem., 229, 763 (1957).
Simpson, E.R. and Boyd, G.S., Biochem. Biophys. Res. Comm., 24, 10 (1966).
Simpson, E.R. and Boyd, G.S., Biochem. J., 103, 17P (1967).
Simpson, E.R. and Boyd, G.S., European J. Biochem. (in press).
Omura, T., Sato, R., Cooper, D.Y., Rosenthal, O. and Estabrook, R.W., Fed. Proc., 24, 1181 (1965).
Omura, T., Sanders, E., Estabrook, R.W., Cooper, D.Y. and Rosenthal, O., Arch. Biochem. Biophys., 117, 660 (1966).