DOES REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE HAVE A DUAL FUNCTION IN STEROID HYDROXYLATION REACTIONS IN ADRENAL MITOCHONDRIA?*

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SUMMARY: The answer is "no".

It has been well established by our work (1, 2, 3) and others (4, 5, 6) that the electron transfer system of adrenal mitochondrial steroid hydroxylases consists of a diaphorase (adrenodoxin reductase), an iron-sulfur protein (adrenodoxin), and a cytochrome (P-450).

This electron transfer system catalyzes the reduction of molecular oxygen by NADPH and produces an unidentified active oxygen of which one atom is incorporated into steroid molecule as hydroxyl group and the other atom is reduced to water. The stoichiometry of oxygen consumption, NADPH utilization and product formation has been thought to be 1:1:1, the same as in some other monooxygenase reactions containing cytochrome P-450 (7, 8).

Shih, Tsong, and Stein (9) and Shih (10) suggested, based upon their studies on the kinetics and the stereospecificity of the utilization of reduced tritiated NADP by the steroid 11-β-hydroxylase from adrenal mitochondria, that NADPH directly reduces the oxygen-oxygen bond by a

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different manner from the NADPH-Adrenodoxin reductase-Adrenodoxin-P-450 system. According to these workers, NADPH serves two distinct roles in the steroid hydroxylation reactions: the reduction of cytochrome P-450 via the electron transfer system and the direct reduction of perferryl ion (Fe^{+++}-O-O^-) to a hydro-peroxo complex (Fe^{++}-O-OH)$^-$. Furthermore, these authors demonstrated that adrenodoxin reductase preferentially removed the H_B-hydrogen of NADPH molecule whereas the deoxycorticosterone-dependent oxidation of NADPH removed the H_A-hydrogen (10).

The conclusion has been questioned by Holtzman (11) who examined the rates of reactions of some hepatic microsomal hydroxylase with ordinary NADPH or 4,4-dideutero NADPH as cofactor. The results showed little isotope effect and led to the conclusion that NADPH is not used to reduce P-450-O_2 complex directly. This conclusion was, however, only tentative as it hinges on certain assumption of the kenictics of the hydroxylase complexes.

In the present communication, we wish to report that the adrenal mitochondrial hydroxylase complexes catalyzing the conversion of deoxycorticosterone to 11-β-hydroxycorticosterone and cholesterol to pregnenolone can function in the absence of NADPH and adrenodoxin reductase but with reduced adrenodoxin as the reductant. It is therefore clear that, if NADPH was capable of direct reducing of the P-450-O_2 complex, it is not an essential step in the hydroxylation.

Table 1 clearly shows that reduced adrenodoxin dependent steroid 11-β-hydroxylation and cholesterol side-chain cleavage (20-α-and 22-ξ-hydroxylation and desmolase) (12) reactions proceeds readily in the absence of NADPH and adrenodoxin reductase. The cytochrome P-450 used was a crude particulate preparation according to Mitani and Horie (13). No contamination of this preparation by pyridine nucleotides could be detected by the fluorescence method using methyl-ethyl-ketone (14). The absolute spectrum of the cytochrome P-450 preparation also indicated

TABLE 1

Experiment No.	Additions					Results
	P-450 mμmole	NADPH μmole	NADH μmole	Reduced Adrenodoxin mμmole	DOC μmole	Corticosterone Formed mμmole
1	0.54	0	0	0	0.90	0
2	0.54	0	0	445	0.90	53
3	0.68	0	0	742	1.05	68
4	0.54	3	0	445	0.90	45
5	2.30	0	0	354	1.05	48
6	2.30	0	3	354	1.05	11
	P-450 mμmole	Cholesterol mμmole		Reduced Adrenodoxin mμmole		Cholesterol Disappeared mμmole
7	2.14	6.8		0		0
8	2.14	6.8		590		1.1
9	2.14	6.8		885		1.7

Adrenodoxin was prepared by the method reported elsewhere (16). Reduced adrenodoxin was prepared by catalytic hydrogenation under strictly anaerobic conditions. Adrenodoxin reductase was prepared by the reported procedure (2) with some modifications. The cytochrome P-450 preparation used was from Step 10 by Mitani and Horie (13). Corticosterone formation was determined by the method of Mattingly (17) and cholesterol side-chain cleavage reaction was assayed by the method previously described (18).

The reaction mixtures for 11-β-hydroxylation contained P-450, deoxycorticosterone, and 0.05 M Tris-HCl pH7.4. Before the addition of reduced adrenodoxin, the mixtures were incubated at 25°C for 15 minutes. The reactions were usually initiated by adding the reaction mixtures into adequate amounts of reduced adrenodoxin and the whole mixtures were gently shaked for 5 minutes.

The reaction mixture for "cholesterol side-chain cleavage" contained: P-450, 26-^{14}C-cholesterol, and 0.05 M Tris-HCl pH7.4. The reactions were carried out the same as above at 25°C.

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the absence of cytochrome b_5. These results clearly demonstrate that the hydroxylation of steroids occurs exclusively via reduced adrenodoxin and cytochrome P-450, without the involvement of NADPH or of cytochrome b_5 (15).

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