## BBA Report

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# Steroid hydroxylations in rat adrenal mitochondria
# III. The ATP–steroid–oxygen stoichiometry of ATP-dependent steroid hydroxylation

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SUMMARY

The energy–steroid–oxygen stoichiometry of ATP-dependent 11-deoxycorticosterone hydroxylation was measured in tightly coupled rat adrenal mitochondria in the presence of KCN and rotenone. The substrate was β-hydroxybutyrate. The results indicate that one molecule of steroid is hydroxylated for each molecule of ATP utilized. It is concluded that in intact rat adrenal mitochondria the stoichiometry of the energy-linked transhydrogenase is one NADPH formed for each high-energy intermediate of oxidative phosphorylation consumed.

In a previous publication[1] we demonstrated a strict energy requirement for $C_{18}$ and 11-β steroid hydroxylations supported by $NAD^+$-linked substrates. In the presence of KCN and rotenone and with either β-hydroxybutyrate or α-ketoglutarate *plus* malonate as substrate, corticosterone (11-β,21-dihydroxy-4-pregnene-3,20-dione) formation required ATP. Mitochondrial steroid hydroxylations supported by $NAD^+$-linked substrates and exogenous ATP are generally believed to occur as follows: NADH and high-energy intermediates (formed by the reversal of oxidative phosphorylation[2,3]) are utilized by the energy-linked transhydrogenase[2,3] for the production of NADPH. The NADPH (*plus* steroid substrate and molecular oxygen) is consumed by the steroid hydroxylation pathway[4,5]. The stoichiometries of energy utilization and of NADPH formation by the energy-linked transhydrogenase have been estimated in intact mitochondria[6] and in submitochondrial particles[2,7] derived from cells that do not produce steroid hormones. The steroid–oxygen stoichiometry for the hydroxylation of 11-deoxycorticosterone has been estimated in adrenal cortex mitochondria[1,8,9]. The overall energy–steroid–oxygen stoichiometry for steroid hydroxylation, however, had not been determined. We report here results that indicate that 1 mole of ATP is utilized

for each mole of 11-deoxycorticosterone hydroxylated by tightly coupled adrenal cortex mitochondria.

Male Sprague-Dawley rats weighing 150–200 g were used in these experiments. The adrenal mitochondrial fractions were prepared by differential centrifugation[1]. Oxygen consumption was measured with a Clark oxygen electrode as previously described[1]. All ATP and steroid additions were made directly to the stirred, full chamber with Hamilton microsyringes. The volumes delivered were calibrated by weighing. ATP solutions were assayed spectrophotometrically by enzymatic methods[10]. Ethanolic solutions of 11-deoxycorticosterone were determined by absorption at 242 nm. Protein was estimated by the biuret method[11]. β-Hydroxybutyric acid dehydrogenase was assayed as described by Lehninger *et al.*[12]. The energy-linked transhydrogenase was measured by the method of Danielson and Ernster[2] in submitochondrial particles prepared from rat adrenal mitochondria by sonication[1] and centrifugation (1 h at 105 000 × *g*).

The oxygen electrode recording shown in Fig. 1 illustrates the ATP dependence of

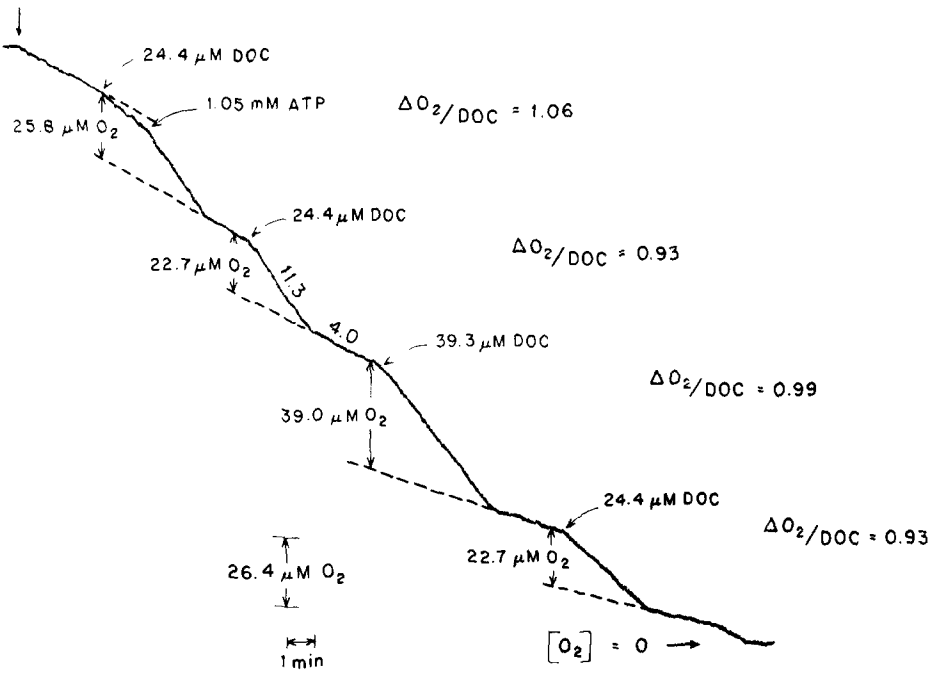


Fig. 1. The oxygen–deoxycorticosterone (DOC) stoichiometry during ATP-activated steroid hydroxylation. The incubation medium contained 40 mM sucrose, 0.8 mM EDTA, 16 mM KCl, 24 mM Tris–HCl, 0.8% bovine serum albumin and 5 mM $P_i$, all at pH 7.4.. D,L-β-Hydroxybutyrate was 10 mM. Rotenone and KCN were 0.01 and 2 mM, respectively. The temperature was 30°. The oxygen electrode chamber volume was 0.95 ml. Mitochondria (1.27 mg mitochondrial protein) were added at the first arrow. Steroid hydroxylation was started by the addition of 24.4 μM 11-deoxycorticosterone. The slow rate of oxygen consumption which ensued was accelerated by the addition of excess ATP. Subsequent 11-deoxycorticosterone additions were made, as indicated, following the complete utilization of the initial 11-deoxycorticosterone addition. The oxygen/11-deoxycorticosterone ratio for each 11-deoxycorticosterone addition is given. The numbers written along the trace are the oxygen consumption rates in nmoles $O_2$/mg mitochondrial protein per min.

oxygen consumption *via* the steroid hydroxylation pathway when respiration due to electron transport over the cytochrome oxidase pathway was inhibited by KCN and rotenone. D,L-β-Hydroxybutyrate was the substrate. A low rate of oxygen consumption (about 4 nmoles/mg mitochondrial protein per min) was observed following the addition of mitochondria. Low rates of KCN-insensitive oxygen consumption appears to be typical of rat[1] and beef[8] adrenal cortex mitochondria. The mechanism of this oxygen consumption is not clear, but since adrenal cortex mitochondria contain two oxygen-requiring pathways, KCN-insensitive oxygen consumption may represent the hydroxylation of endogenous steroids, presumably cholesterol[13]. The addition of a low concentration of 11-deoxycorticosterone often caused a slight increase in the rate of oxygen consumption, apparently due to the non-energy-linked transhydrogenase. Addition of an excess of ATP gave about a 3-fold increase in the rate of oxygen consumption. This enhanced activity continued until all of the added 11-deoxycorticosterone had been hydroxylated; then oxygen consumption returned to the initial low rate. Subsequent 11-deoxycorticosterone additions repeated this cycle until the oxygen of the medium had been exhausted. The oxygen–steroid stoichiometry during this steroid hydroxylation is indicated by the ratio of the concentration of oxygen consumed to the concentration of 11-deoxycorticosterone added. These values were close to 1: In seven determinations a mean ratio value (± S.E.) of 1.07 ± 0.05 nmoles oxygen consumed per nmole of 11-deoxycorticosterone added was observed.

The oxygen electrode recording shown in Fig. 2 shows an experiment which differs from that of Fig. 1 in that the first addition was excess 11-deoxycorticosterone. Steroid hydroxylation was stimulated by the addition of a low concentration of ATP. Oxygen consumption due to steroid hydroxylation continued until all of the added ATP had been utilized. This cycle could be repeated by subsequent ATP additions. Oligomycin abolished the response to the next ATP addition. Steroid hydroxylation, however, could be started again by the addition of malate, a substrate that is able to supply NADPH by an energy-independent reaction in rat adrenal cortex mitochondria[1]. The energy–oxygen stoichiometry is indicated by the ratio of the concentration of oxygen consumed to the concentration of ATP added. Again, this ratio is close to 1. In 16 similar experiments performed with three different mitochondrial preparations a mean ratio value (± S.E.) of 0.75 ± 0.03 nmoles of oxygen consumed per nmole of ATP added was observed. Since the amount of oxygen consumed is equivalent to the amount of steroid hydroxylated (Fig. 1), we conclude that 0.75 nmoles of 11-deoxycorticosterone were hydroxylated for each nmole of ATP added. This value is sufficiently close to 1 to warrant the conclusion that 1 mole of ATP is required for the hydroxylation of 1 mole of 11-deoxycorticosterone. A mean ratio value closer to 1 was not expected for we have already shown that during the forward reaction of oxidative phosphorylation, these mitochondrial preparations show a phosphorylation efficiency of about 0.8 ATP's per energy-coupling site[1]. Since the reverse reaction (the formation of high-energy intermediates from ATP) is unlikely to be more efficient than the forward reaction, mean ratio values for the stoichiometric relationship between 11-deoxycorticosterone and ATP consumption should not be greater than about 0.8 if one high-energy intermediate is involved.

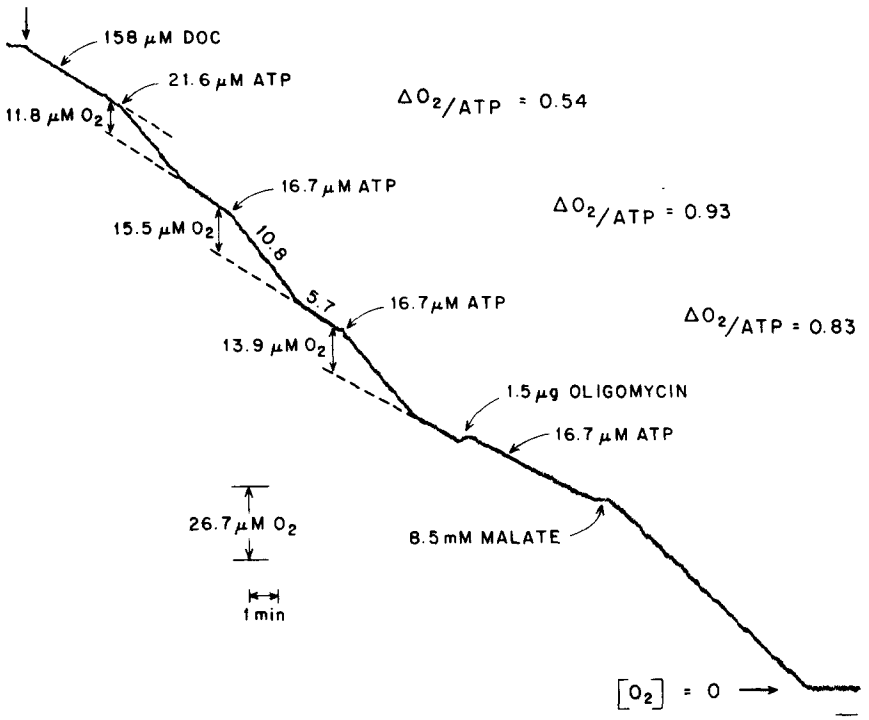


Fig. 2. The oxygen–ATP stoichiometry during ATP-activated steroid hydroxylation. The contents of the incubation medium and conditions are as described in the legend to Fig. 1. Mitochondria (1.27 mg mitochondrial protein) were added at the first arrow. An excess of 11-deoxycorticosterone (DOC; 158 μM) was then added. Active oxygen consumption occurred after the first ATP addition and continued until the ATP was exhausted. Subsequent ATP additions and addition of oligomycin and malate are as indicated. The oxygen/ATP ratio for each ATP addition is given. The numbers written along the trace are the rates of oxygen consumption in nmoles $O_2$/mg mitochondrial protein per min.

We interpret these findings to mean that during energy-dependent steroid hydroxylation 1 ATP molecule is utilized by the energy-linked transhydrogenase for each NADPH formed[2,6,7]. This interpretation follows from data in the literature if it is assumed that all of the added ATP is used to drive the energy-linked transhydrogenase. In separate experiments (not shown here) we have confirmed that the β-hydroxybutyric acid dehydrogenase of rat adrenal mitochondria is membrane bound and active with $NAD^+$ but not with $NADP^+$ either in the presence or absence of ATP. These results agree with the report of Lehninger *et al.*[12]. Also, we find that rat adrenal submitochondrial particles contain an active energy-linked transhydrogenase that is dependent on NADH, ATP, $Mg^{2+}$, and $NADP^+$. This finding confirms the observation of Oldham *et al.*[3] in bovine adrenal cortex submitochondrial particles.

The assumption that all of the added ATP serves to drive the energy-linked transhydrogenase is more difficult to verify. Papa *et al.*[6] have commented on the problems involved. There is a lack of information with regard to the various ATP-utilizing reactions that might occur simultaneously with the energy-linked transhydrogenase. In our experiments, as in those of Papa *et al.*[6], D,L-β-hydroxybutyrate was

the substrate used; therefore, a portion of the ATP may have been utilized to form the coenzyme A ester of the dextrorotatory enantiomer[14,15]. It is possible that ATP utilization by this reaction is responsible, at least in part, for the low oxygen to ATP ratio often observed following the initial ATP addition (Fig. 2). The oligomycin sensitivity of ATP-dependent steroid hydroxylation indicates that the formation of high-energy intermediates is obligatory. While there is some evidence that long-chain fatty acids may be activated by high-energy intermediates in rat liver[16] and in bovine brain[17] mitochondria, other data favor an oligomycin-insensitive ATP activation of fatty acids within the intramitochondrial space[18,19]. In addition, we are not aware of any data indicating that activation of L-(+)-β-hydroxybutyrate will proceed through the high-energy intermediates of oxidative phosphorylation. The consistently high 11-deoxycorticosterone/ATP ratios observed in these experiments are perhaps the best evidence that other ATP-consuming reactions present in rat adrenal mitochondria utilized little or none of the ATP.

Papa *et al.*[6] found that $P_i$ formation from added ATP was stoichiometrically related to $NADP^+$ consumption by the energy-linked transhydrogenase in KCN-inhibited, intact rat liver mitochondria supported by D,L-β-hydroxybutyrate. Although the range in the individual values was fairly wide (0.24–1.05 and 0.62–1.43), the mean $NADP^+/P_i$ ratio values were 0.52 and 0.89, respectively, 6 and 10 sec after the addition of ATP *plus* substrate. In our experiments a relatively narrow range (0.54–0.99, $N = 16$) in the individual oxygen/ATP ratio values was observed. The mean value (0.75) agrees well with the mean values of Papa *et al.*[6].

Recently we demonstrated that steroid hydroxylation supported by α-ketoglutarate *plus* malonate or β-hydroxybutyrate competed with oxidative phosphorylation for high-energy intermediates[20]. We assumed that one of the ATP's lost during the competitive reactions resulted from the high-energy intermediate consumed by the energy-linked transhydrogenase. These data support that assumption and emphasize the crucial role played by the energy-linked transhydrogenase in steroid hydroxylation supported by $NAD^+$-linked substrates.

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