SPIN STATE CHANGES IN CYTOCHROME *P*-450 ASSOCIATED WITH CHOLESTEROL SIDE CHAIN CLEAVAGE IN BOVINE ADRENAL CORTEX MITOCHONDRIA

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1. Introduction

Cholesterol side chain cleavage and steroid 11β-hydroxylation are both associated with the cytochrome *P*-450 electron transport system of bovine adrenal cortex mitochondria [1, 2]. Cholesterol side chain cleavage (SCC) activity is enhanced by the action of ACTH [3] while subsequent steps in the production of corticosteroids, including the 11β-hydroxylase activity, remain unaffected.

Recently the adrenal mitochondrial cytochrome *P*-450 associated with cholesterol side chain cleavage has been separated from the cytochrome *P*-450 associated with 11β-hydroxylase activity [4]. The former fraction is isolated as a high spin form of the oxidised cytochrome (λ_{max} 392 nm) whereas the latter is isolated as a low spin form (λ_{max} 416 nm) of the oxidised cytochrome.

These studies have led to a clarification of spectral changes seen in intact bovine adrenal cortex mitochondria on addition of malate or succinate [5]. In particular a rapid high to low spin state transition in SCC-cytochrome *P*-450, has been demonstrated to occur without valency change, and which is related to the conversion of cholesterol to pregnenolone.

2. Methods

Bovine adrenal cortex mitochondria were prepared as described by Cammer and Estabrook [6]. Soluble SCC-cytochrome *P*-450 was prepared as previously described [7]. Optical spectra were obtained with an Aminco-Chance split beam dual-wavelength spectrophotometer and EPR spectra were obtained with a Varian E4 spectrometer at - 175°. Fluorescence changes were measured using a Hitachi-Perkin Elmer MPF-2A spectrofluorimeter fitted with a Servoscribe RE511 recorder.

Cholesterol side chain cleavage was initiated by addition of malate to intact bovine adrenal cortex mitochondria (protein = 4–5 mg/ml) suspended in the following medium: 250 mM sucrose, 20 mM KCl, 15 mM triethanolamine hydrochloride, 10 mM potassium phosphate, 5 mM $MgCl_2$, pH 7.3. Cholesterol side chain cleavage activity was measured in the soluble SCC-cytochrome *P*-450 preparation by using an NADPH generator, and NADPH–cytochrome *P*-450 reductase as described previously [4]. Pregnenolone was assayed after the steroids were extracted and separated on TLC as previously described [1]. 7α-^{3}H-Pregnenolone (Radiochemical Centre, Amersham) was added as an internal standard (0.06 μCi, 5000 μCi/mole). The region of the plates corresponding to 7-^{3}H-pregnenolone was eluted with chloroform–methanol (1:1), taken to dryness and dissolved in 0.1 ml ethanol followed by 2 ml 0.1 M triethanolamine hydrochloride buffer pH 7.2. Pregnenolone was estimated by addition of a steroid-3β-ol dehydrogenase preparation from Pseudomonas testeroni (Sigma Chemical Co., St. Louis) [8] and an excess of NAD^+ (1 mM). The formation of NADH was

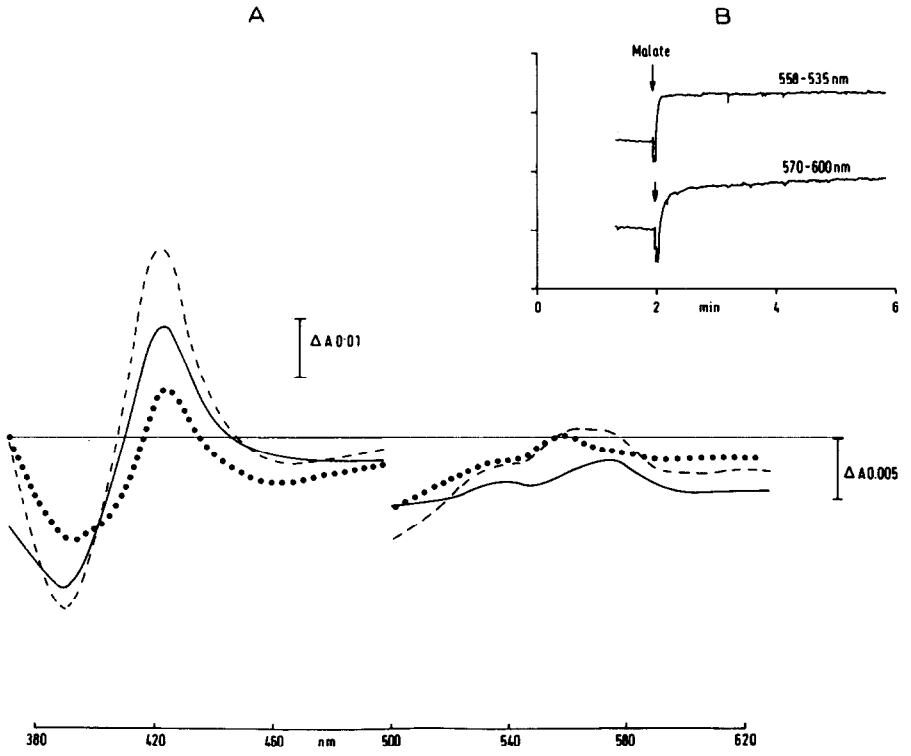

Fig. 1 (A). Difference spectra of bovine adrenal cortex mitochondria. —— 42 μM pregnenolone in sample cuvette. Each cuvette also contained 1.2 μM 2α-cyano-4,4,17α-trimethyl 17β-hydroxy-5-androsten-3-one (cyanoketone) [9]. - - - - 3.3 mM malate in sample cuvette. Each cuvette also contained 3 μg/ml rotenone to prevent reduction of respiratory cytochromes. . . . . 3.3 mM malate in sample cuvette and 42 μM pregnenolone in both cuvettes. Each cuvette also contained 1.2 μM cyanoketone and 3 μg/ml rotenone. Protein concentration 2.9 mg/ml. (B) Kinetics of reduction of *b*-type cytochrome and high → low spin state transition of cytochrome *P*-450 on addition of malate. Conditions as for sample cuvette of 1A.

followed fluorimetrically and quantitated with standard pregnenolone solutions. The percentage recovery of pregnenolone from the reaction mixture was estimated from the recovery of 7-^{3}H-pregnenolone which was generally 60–80%. All experiments were conducted at 22°.

3. Results and discussion

Fig. 1A shows the type II difference spectrum obtained by addition of pregnenolone to bovine adrenal cortex mitochondria (solid line; λ_{max} 422, 535, 574 nm). Fig. 1A also shows the difference spectrum obtained by addition of malate in the presence of rotenone (dashed line). This differs from the pregnenolone difference spectrum chiefly in the presence of an additional absorption peak at about 560 nm. Thus malate, like pregnenolone, appears to convert high spin cytochrome *P*-450 to a low spin state. The low spin component formed by addition of malate was largely balanced out by addition of pregnenolone to both cuvettes leaving a residual spectrum which was predominantly the reduced minus oxidised difference spectrum of a *b*-type cytochrome (fig. 1A, dotted line; λ_{max} 425, 534, 558 nm).

Fig. 1B shows a comparison of the kinetics of reduction of the *b*-type cytochrome (ΔA 558–535 nm) and formation of the type II change (ΔA 570–600 nm) on addition of malate. These are clearly different, the lower trace being apparently

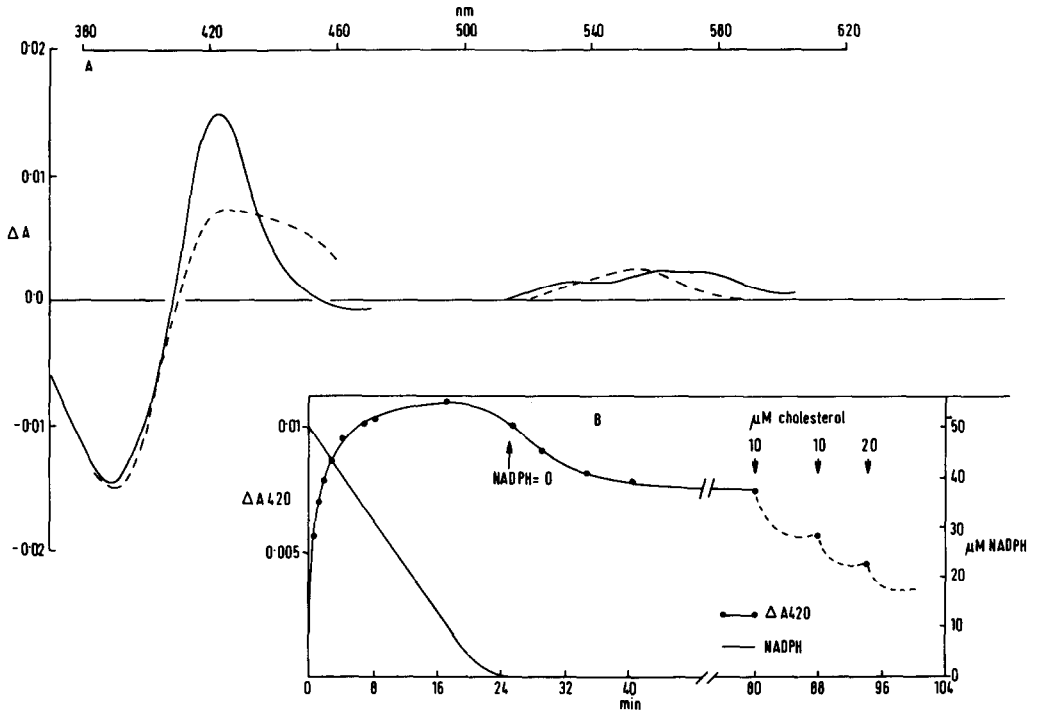

Fig. 2 (A). Difference spectra of solubilised SCC-cytochrome *P*-450 preparation. —— NADPH generating system in sample cuvette. Each cuvette contained 0.5 nmoles/ml SCC-cytochrome *P*-450 and enough NADPH–cytochrome *c* reductase to reduce 120 nmoles cytochrome *c*/ml/min in 20 mM potassium phosphate buffer pH 7.2. ---- Reduced minus oxidised difference spectrum of SCC-cytochrome *P*-450. The sample cuvette was reduced with dithionite for 30 min, the reference cuvette for 2 min. Each cuvette contained 0.5 nmoles/ml SCC-cytochrome *P*-450 in 20 mM potassium phosphate buffer pH 7.2. (B) Formation and disappearance of the absorbance change (420–408 nm) produced by addition of 50 μM NADPH to solubilised SCC-cytochrome *P*-450 in the sample cuvette. Each cuvette contained NADPH–cytochrome *c* reductase as indicated in fig. 2 (A). The disappearance of NADPH was followed in a parallel experiment in the fluorimeter. At the times indicated, cholesterol was added to both cuvettes.

a composite of a fast step and a slow step which have not been further resolved.

These spectral changes can be more clearly defined by comparison with spectral changes on the soluble SCC-cytochrome *P*-450 preparation. Addition of NADPH to this preparation in the presence of NADPH-cytochrome *P*-450 reductase, resulted in a difference spectrum similar in magnitude and characteristics to that induced by saturating amounts of pregnenolone (fig. 2A, solid line; λ_{max} 422, 534, 575 nm), although there was again a small contribution from reduction of a *b*-type cytochrome (λ_{max} 560 nm) [7]. The *b*-type cytochrome was much more rapidly reduced by dithionite than SCC-cytochrome *P*-450, [7]. This permitted a reduced-oxidised difference spectrum for SCC-cytochrome *P*-450 to be obtained (fig. 2B, dashed line) which was clearly distinguished from the NADPH-induced difference spectrum, particularly in the α and β bands.

Further evidence that malate was inducing a change of the spin state of SCC-cytochrome *P*-450 in intact mitochondria was obtained from EPR spectra (fig. 3). After reaction for one minute malate caused a 35 percent intensification of the signal due to low spin cytochrome *P*-450 (S_m = 2.42, g = 2.26, S_m = 1.91).

This change in spin state appears to be reflected in simultaneous changes in oxygen uptake and pregnenolone formation following addition of malate to intact adrenal mitochondria, in the presence of the

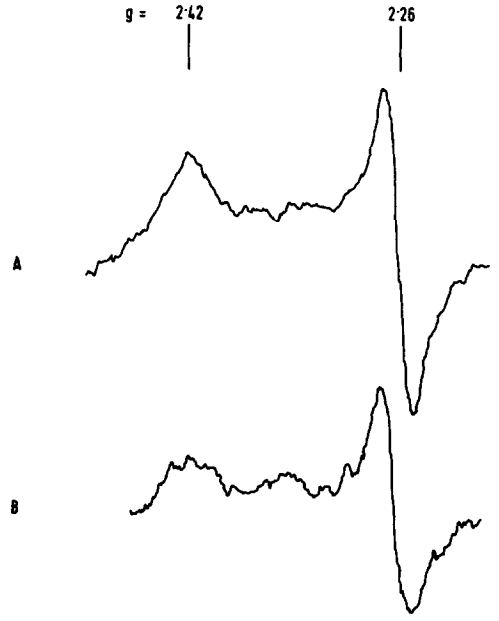

Fig. 3. EPR spectra of bovine adrenal cortex mitochondria at −175°. (A) after 1 min incubation with 6 mM malate in the presence of 3 μg/ml rotenone. (B) no additions. The mitochondrial suspensions were gassed with oxygen before and during the incubations. Protein concentration 21 mg/ml.

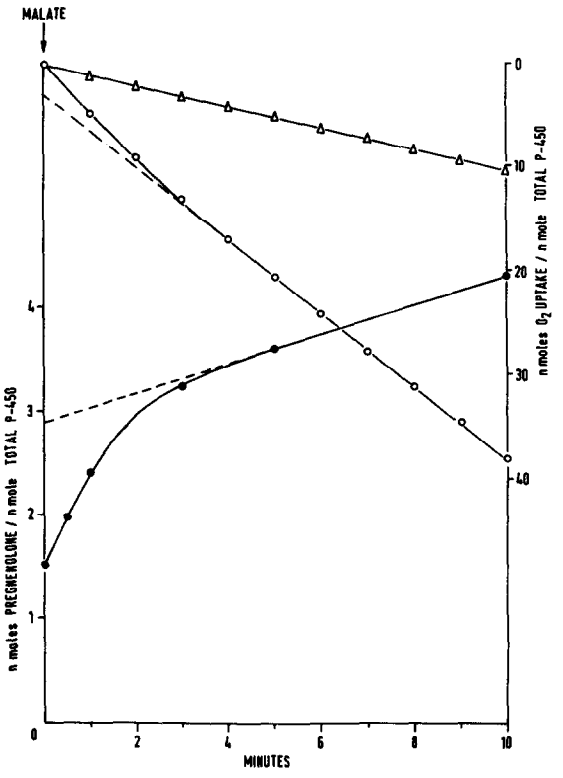

Fig. 4. Pregnenolone formation and oxygen uptake on addition of malate to bovine adrenal cortex mitochondria in the presence of rotenone and cyanoketone. ●——● Pregnenolone formation. ○——○ Oxygen uptake in the presence of malate (3.3 mM). △——△ Oxygen uptake in the absence of malate. Rotenone concentration 3 μg/ml; cyanoketone concentration 1 μM; protein concentration 5.2 mg/ml.

3β-ol dehydrogenase inhibitor, cyanoketone (2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androsten-3-one) [9] and rotenone (fig. 4). Malate initiated a rapid oxygen uptake lasting 2–3 min which was followed by a slower steady rate. The rapid phase involved uptake of about 3 nmoles oxygen/nmole total cytochrome *P*-450 in excess of the steady rate, and correlated with a rapid conversion of cholesterol to pregnenolone. This rate decreased to a much slower steady rate after 2–3 min. This initial phase produced about 1.4 nmoles pregnenolone/nmole cytochrome *P*-450. A rather similar time course for pregnenolone formation has been observed in rat adrenal mitochondria by Koritz and Kumar [10]. The approximate stoichiometry of 2 O_2/pregnenolone suggests that the initial oxygen uptake is derived from the conversion of cholesterol to pregnenolone.

In further experiments, both phases of malate-induced oxygen uptake were found to be inhibited by 100 μg/ml aminoglutethimide, which inhibits cholesterol side chain cleavage [11]. Consequently this oxygen utilisation must result from reoxidation of reduced SCC-cytochrome *P*-450. The predominance of the oxidised state after addition of malate must be due to the faster rate of reoxidation. The correlation between spectra, oxygen uptake and pregnenolone formation indicates that each oxidation-reduction cycle of the high spin form of SCC-cytochrome *P*-450 contributes almost quantitatively to the production of pregnenolone. However, the steady rate of oxygen uptake which corresponds to SCC-cytochrome *P*-450 in a predominantly low spin steady state, involves very little pregnenolone formation, and probably arises mainly from unproductive formation of hydrogen peroxide, or other peroxidative reactions.

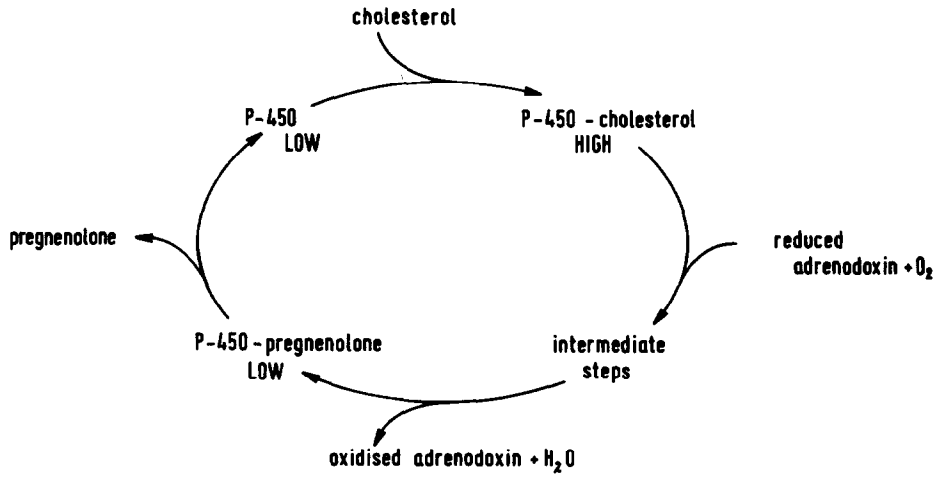

Fig. 5. Possible events taking place during cholesterol side chain cleavage.

The sequence of changes following the addition of NADPH to soluble SCC-cytochrome *P*-450 are shown in fig. 2B. This preparation contains about 2.5 nmoles cholesterol/nmoles cytochrome *P*-450 [7]. Low spin cytochrome *P*-450 was formed very rapidly, then more slowly. NADPH was consumed at a constant rate until the concentration fell below 6 μM when the rate of NADPH consumption and the magnitude of the difference spectrum both began to decrease. The difference spectrum continued to decrease very slowly after all the NADPH had been consumed finally reaching a limit of 65 percent of the maximum spectrum. This residual difference spectrum however was further halved by addition of 40 μM cholesterol.

The NADPH-induced change in the spin state of SCC-cytochrome *P*-450 is partly due to the low spin complex formed by pregnenolone. However, titration of adrenal cortex mitochondria with pregnenolone in the absence of reducing equivalents showed that a maximum of 30 percent of the observed low spin SCC-cytochrome *P*-450 can be attributed to the pregnenolone complex. Thus the NADPH-induced change in the spin state seems to be due mainly to a perturbation of the spin state equilibria (fig. 5), by the oxidation-reduction cycle. When the supply of NADPH to the soluble SCC-cytochrome *P*-450 was exhausted a partial reversal to the high spin state was observed (fig. 2B). Addition of low concentrations of cholesterol produced a further reconversion of SCC-cytochrome *P*-450 to the high spin state. An explanation of the steady state change in spin state is that the high spin cholesterol complex is converted to pregnenolone and low spin SCC-cytochrome *P*-450, in a relatively fast step, while the latter combines only slowly with cholesterol to reform the active complex. The low spin cytochrome comprises both free cytochrome and the pregnenolone complex. In fig. 5 the various states of SCC-cytochrome *P*-450 have been related to cholesterol side chain cleavage.

These results emphasise that the rate of cholesterol side chain cleavage in adrenal mitochondria may be limited by the rate of transport of cholesterol to SCC-cytochrome *P*-450 from other mitochondrial sites, in addition to possible inhibition by pregnenolone [10]. The pool of cholesterol which can be metabolised rapidly in bovine adrenal cortex mitochondria appears to be about 3 nmoles cholesterol per nmole SCC-cytochrome *P*-450. Clearly, as pregnenolone formation is accelerated by ACTH in vivo, then cholesterol transport could be an important limiting process for in vivo cholesterol side chain cleavage activity in adrenal cortex mitochondria.

Acknowledgements

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