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PURIFICATION OF ADRENODOXIN REDUCTASE FROM BOVINE ADRENAL CORTEX MITOCHONDRIA BY AFFINITY CHROMATOGRAPHY. PROPERTIES OF STEROID HYDROXYLASE SYSTEMS RECONSTITUTED FROM ADRENODOXIN REDUCTASE, ADRENODOXIN AND MEMBRANOUS CYTOCHROME P-450$_{11\beta}$

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SUMMARY

Adrenodoxin reductase from bovine adrenal cortex mitochondria was purified to homogeneity by affinity chromatography on 2',5'-ADP-Sepharose 4B. The purified adrenodoxin reductase was reconstitutively active in the presence of NADPH and adrenodoxin and with either cytochrome *c* or cytochrome P-450 as electron acceptor. In sonicated cytochrome P-45-containing mitochondrial membranes reconstituted with adrenodoxin reductase and adrenodoxin, the content of cytochrome P-450 and level of reduction of cytochrome P-450 was not altered as compared to intact mitochondria. In contrast, hydroxylation of deoxycorticosteron was inhibited, suggesting that a component of cytochrome P-450 or an hypothetical factor required for hydroxylation of steroids by membrane-bound cytochrome P-450, was impaired.

INTRODUCTION

Adrenal cortex mitochondria contain the hydroxylase systems responsible for side-chain cleavage of cholesterol and 11β- and 18-hydroxylation of various steroids [*cf.* 1 and 2]. All three systems share the two common electron carriers, adrenodoxin reductase (EC 1.6.7.1) and adrenodoxin, which both have been purified by conventional methods and characterized extensively [3–12], and which mediate electrons from NADPH to the two cytochrome P-450 types of heme proteins. The cytochrome P-450 catalyzing side-chain cleavage of cholesterol (cytochrome P-450$_{scc}$) and that catalyzing 11β- and 18-hydroxylation (cytochrome P-450$_{11\beta}$) have also been isolated recently [13–18]. It now appears established that 11β- and 18-hydroxylation are catalyzed by the same cytochrome P-450 [17, 19, 20]. However, the properties of the different hydroxylase systems reconstituted from the individual purified components have not been thoroughly investigated. Since all components of the mitochondrial cytochrome P-450 systems normally are bound to the mitochondrial inner membrane[1, 2] it appears necessary to reconstitute the isolated components into liposomes in order to properly characterize the systems. Although nonvesicular, phospholipid-activated, systems have been described [21], it was only very recently that a cytochrome P-450$_{scc}$ system reconstituted in liposomes was described [22].

In the present investigation adrenodoxin reductase and adrenodoxin were reconstituted with mitochondrial inner membrane fragments devoid of adrenodoxin reductase and adrenodoxin but containing cytochrome P-450. To ensure proper recombination of active components, adrenodoxin reductase was purified by affinity chromatography on 2',5'-ADP-Sepharose CL-4B to homogeneity. The results indicate that the purified adrenodoxin reductase was reconstitutively active and mediated reduction of cytochrome P-450$_{11\beta}$ as well as hydroxylation of deoxycorticosterone. However, with a system reconstituted from adrenodoxin reductase, adrenodoxin and mitochondrial membranes exposed to prolonged sonication, reduction of cytochrome P-450 took place readily whereas hydroxylation of deoxycorticosterone was not observed. This finding suggests that a component of cytochrome P-450$_{11\beta}$ or an unknown additional factor was impaired, which is essential for 11β-hydroxylation of deoxycorticosterone but not for reduction of cytochrome P-450.

MATERIALS AND METHODS

Bovine adrenal cortex mitochondria were prepared as described earlier [23], and stored at −20°C at a

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protein concentration of 30 mg/ml. About 1 g of mitochondria was sonicated and centrifuged overnight essentially as described by Foster and Wilson[6] except that 3 mM EDTA and 1 mM dithiothreitol was added to the medium. Ammonium sulphate (saturated at 4°C, and adjusted with tris base to pH 7.5) was added to the supernatant to 39% saturation. After incubation for 20 min the mixture was centrifuged for 30 min at 100,000 *g* (average) with a Beckman 50 Ti rotor and the resulting pellet discarded. The ammonium sulphate concentration in the supernatant was then increased to 60% saturation, the centrifugation step repeated, and the resulting pellet suspended in a few ml of 20 mM sodium tricine (pH 8.0) containing 2 mM EDTA and 1 mM dithiothreitol. After dialysis overnight against 50 ml of the above buffer, the crude adrenodoxin reductase was applied to a DEAE-Sepharose CL-6B column (1.5 × 15 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.4) washed with 2 column volumes of buffer, and eluted with a linear potassium phosphate gradient from 10 to 100 mM (pH 7.4, total volume 400 ml). The most active fractions were pooled, concentrated ten times, diluted 10 fold with 20 mM tricine (pH 8.0), 3 mM EDTA and 1 mM dithiothreitol and reconcentrated 10 fold in an Amicon ultrafiltration cell (Amicon Corp., Lexington, MA U.S.A.) equipped with a PM 10 filter. In the final affinity chromatography step the concentrate was applied to a 2',5'-ADP-Sepharose CL-4B (N^6-(6-aminohexyl)-adenosine-2',5'-bisphosphate-Sepharose CL-4B column (1 × 3 cm) equilibrated with 20 mM sodium tricine (pH 8.0), 3 mM EDTA and 1 mM dithiothreitol. The adrenodoxin reductase bound as a sharp yellow band at the top of the gel. A substantial amount of inactive protein passed through the column upon washing with two volumes of equilibration buffer. Homogeneous adrenodoxin reductase was then eluted by inclusion of 1 ml of 50 mM 2'-AMP (pH 8.0) and stored at −70°C or below. The 2'-AMP present in the preparation was not routinely removed since it did not interfere with the assay of the enzyme. However, if necessary the bulk of the nucleotide can be removed by ultrafiltration. Prior to the addition of 2'-AMP, the bound adrenodoxin reductase was occasionally washed further with equilibration buffer containing 50 mM KCl. This treatment spread the yellow band without eluting the enzyme but did not increase the specific activity of the final preparation significantly. All steps were carried out at 4°C.

Homogenous adrenodoxin from beef adrenal cortex mitochondria in high yield was purified from a cholate extract containing ammonium sulphate to 32% saturation, as described by Takemori *et al.*[24]. Ammonium sulphate (saturated at 4°C) was added to 60% saturation after which the mixture was centrifuged for 30 min at 100,000 *g* (average). The supernatant was dialyzed overnight against 20 mM sodium tricine (pH 8.0) and 3 mM EDTA. The dialysate was then adsorbed on a DEAE-Sepharose CL-6B column (2 × 30 cm equilibrated with mM sodium tricine (pH 8.0) and 3 mM EDTA) and eluted with a gradient of 0 to 1 M sodium chloride in the equilibration buffer in a total volume of 600 ml. Adrenodoxin-containing fractions were pooled, concentrated to about 2 ml in an Amicon cell (UM2 filter) and chromatographed on a Sephadex G-100 column (1.5 × 100 cm) in the presence of 20 mM sodium tricine (pH 8.0), 3 mM EDTA and 0.5 M potassium chloride. Adrenodoxin was concentrated and finally desalted on a Sephadex G-25 column (2 × 30 cm), equilibrated with 20 mM sodium tricine (pH 8.0) and 3 mM EDTA and stored at −70°C or below. All steps were carried out at 4°C. Media used in column chromatography were degassed and saturated with nitrogen. Adrenodoxin prepared by this method was homogenous on sodium dodecyl sulphate polyacrylamide gels and gave an absorbance ratio at 415 nm and 280 nm (A_{415}/A_{280}) of at least 0.6. The yield was about 3 mg per g mitochondrial protein.

Mitochondrial fragments essentially devoid of adrenodoxin reductase and adrenodoxin but containing cytochrome P-450, were obtained by centrifuging mitochondria for 10 min at 100,000 *g* that previously had been exposed to treatment with a Ribi Cell Fractionator (Sorwall model RF-1) at a pressure of 20,000 psi, followed by sonication for at least 90 seconds with a Branson sonifier, model S-125 at 4A, in the presence of 50 mM Tris–HCl buffer (pH 7.0) containing 0.25 M sucrose and 0.5 M potassium chloride. During sonication the temperature was not allowed to exceed 10°C. The pellet obtained was resuspended in the same buffer at a protein concentration of 20 mg/ml, in the absence of potassium chloride.

Protein was measured by the method of Bradford[25]. NADPH-cytochrome *c* reductase activity was measured essentially according to Foster and Wilson[6] employing 20 mM sodium tricine (pH 7.4) as buffer. Absorption spectra were recorded with a Cary 17 spectrophotometer. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and molecular weight determination was carried out on a gradient (7.5–15%) slab polyacrylamide gel according to Alvares and Siekevitz[26]; bovine serum albumin, catalase, ovalbumin, yeast alcohol dehydrogenase, phosphorylase a (rabbit), cytochrome *c*, and trypsin inhibitor were employed as reference proteins. Hydroxylation of deoxycorticosterone to corticosterone was assayed spectrophotometrically as described previously [27]. Hydroxylation of [^{14}C]-deoxycorticosterone was carried out essentially as described earlier [27]. N^6-(6-aminohexyl)-adenosine-2',5'-bisphosphate-Sepharose CL-4B (2',5'-ADP-Sepharose 4B) and DEAE-Sepharose CL-6B were purchased from Pharmacia Chem. Co. (Uppsala, Sweden). 21-Hydroxy-4-[4-^{14}C]pregnene-3,20-dione (deoxycorticosterone, specific radioactivity 59.8 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, England. Other biochemicals were obtained from Sigma Chem. Co. (St. Louis,

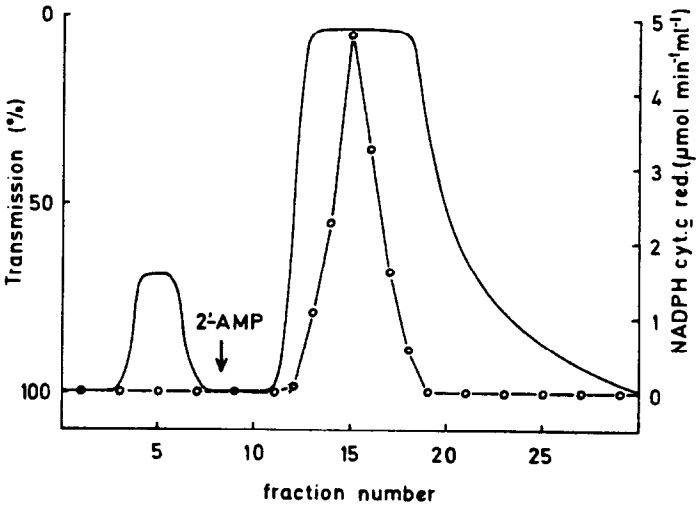

Fig. 1. Elution profile of adrendoxin reductase from 2',5'-ADP-Sepharose 4B. Elution of adrenodoxin reductase was initiated by the inclusion of 50 mM 2'AMP in the medium as described in Methods. O——O, NADPH-cytochrome *c* reductase activity; ——, absorbance at 254 nm. The second absorbance peak is due mainly to 2'-AMP.

Mo., U.S.A.) or Boehringer GmbH (Mannheim, W. Germany).

RESULTS

Purification and properties of adrenodoxin reductase

Previous investigations of the reconstitutive properties of adrenodoxin reductase have indicated that, in the presence of adrenodoxin and adrenodoxin reductase purified by conventional methods [6], reduction of cytochrome P-450 by NADPH was less reproducible (J. Montelius and J. Rydström, unpublished). Since this lack of reproducibility appeared to be due to the adrenodoxin reductase, a highly simplified purification procedure was introduced, which was based on binding of the active enzyme to a 2',5'-ADP-Sepharose CL-4B gel. Similar procedures have been used previously for the purification of various reductases [28]. Adrenodoxin reductase bound readily to the gel and was then eluted specifically by a pulse of 50 mM 2'-AMP (Fig. 1). As expected, 2-AMP could be replaced by less than 1 mM NADP(H) or 2',5'-ADP; salts, e.g., potassium chloride at concentrations above 200 mM also eluted the enzyme (not shown). The specific activity of the adrenodoxin reductase normally varied between 8 and 10 μmol/min/mg protein although activities of 12 μmol/min/mg protein have been observed. Crude adrenodoxin reductase that had not been partially purified on DEAE-Sepharose also bound to the 2',5'-ADP column but with an apparently lowered affinity, which seemingly resulted in a very low capacity of the column for the enzyme. Tentatively, these results may be explained by the presence of adrenodoxin which was removed in the DEAE-Sepharose step. Reduction of adrenodoxin reductase with sodium dithionite during binding to the 2'-5'-ADP column did not increase the affinity of the enzyme for the ligand, as was suggested from previous kinetic studies [10]. The complete purification procedure is summarized in Table 1.

High resolution polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (Fig. 2) indicated that the reductase preparation was homogenous and completely devoid of contaminants. The minimal molecular weight was estimated to be 50,000 daltons which is an agreement with the value of Foster and Wilson[6]. Purified adrenodoxin reductase revealed a resolved absorption spectrum in the visible region (Fig. 3) which is typical of a flavopro-

Table 1. Purification of adrenodoxin reductase from bovine adrenal cortex mitochondria

	Protein (mg)	S.A. (μmol/min/mg prot.)	Total activity (μmol/min)	Purification (times)	Yield (%)
Sonic extract	42.2	0.54	22.75	1.0	100
39-60P	22.6	0.94	21.34	1.8	93
Dialyzate	17.0	1.11	18.83	2.1	82
DEAE-Sepharose pool	2.9	2.46	7.11	4.6	31
Affinity chrom. pool	0.49	8.52	4.14	15.8	18

The enzyme was assayed as NADPH-cytochrome *c* reductase in the presence of saturating concentrations of adrenodoxin (*cf.* Methods).

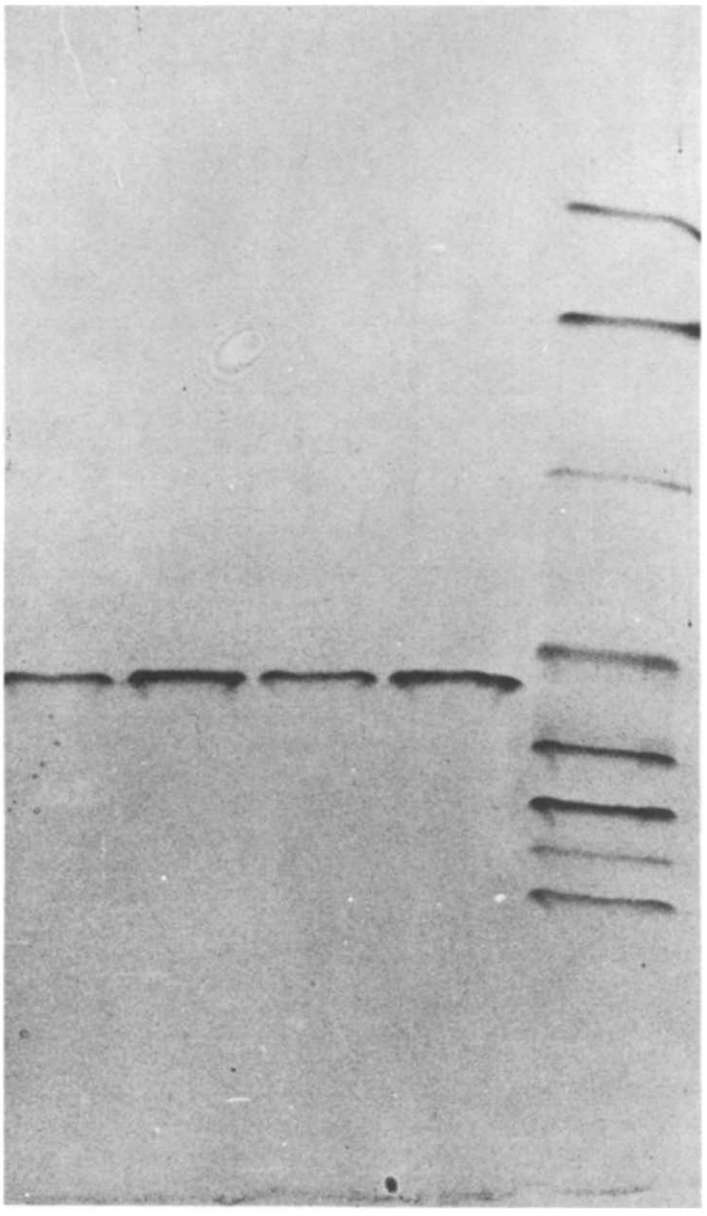

Fig. 2. Polyacrylamide gel electrophoresis of purified adrenodoxin reductase in the presence of sodium dodecyl sulphate. The slab gel contained five different protein samples which were (from left to right): 5 μg adrenodoxin reductase (preparation 1), 10 μg adrenodoxin reductase (preparation 1), 5 μg adrenodoxin reductase (preparation 2), 10 μg adrenodoxin reductase (preparation 2) and 50 μg reference proteins. Preparation 1 and preparation 2 represent two different preparations of adrenodoxin reductase and demonstrate the reproducibility of the purification method described. Migration direction was from bottom to top. The gel was stained with Coomassie Brilliant Blue G-250.

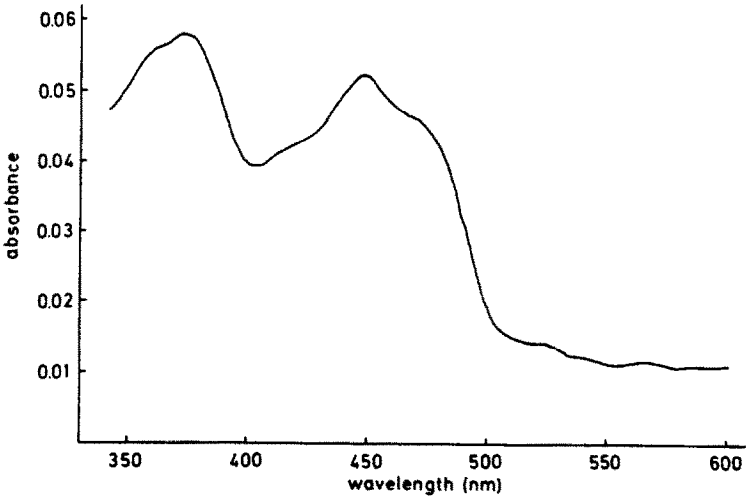

Fig. 3. Absorbance spectrum of homogenous adrenodoxin reductase. The concentration of adrenodoxin reductase was 0.21 mg protein/ml. Medium was 20 mM sodium tricine (pH 8.0).

tein and similar to those described earlier [6, 10]. Due to the presence of residual 2'-AMP in the reductase preparation (even after repeated Sephadex or filtration steps) a reliable UV spectrum could not be recorded. The spectrum showed characteristic peaks at 378 nm and 455 nm, the latter with a shoulder at 477 nm.

Reconstitution of NADPH-cytochrome c *reductase, reduction of cytochrome P-450 by NADPH in the presence of CO, and 11β-hydroxylase*

The capability of the homogeneous adrenodoxin reductase and adrenodoxin to support reduction of cytochrome *c* and cytochrome P-450 as well as hydroxylation of steroids by NADPH was tested with a reconstituted system composed of adrenodoxin reductase, adrenodoxin and the electron acceptors cytochrome *c* or cytochrome P-450. As shown in Fig. 4, reduction of cytochrome *c* (present in excess) by NADPH revealed that optimal rates of reduction of cytochrome *c* were obtained at approximately the same concentration of adrenodoxin at varying concentrations of adrenodoxin reductase, indicating that, under these conditions adrenodoxin behaved as a soluble substrate of the reductase rather than formed a tight equimolar complex with reductase (*cf.* 5). With cytochrome P-450 as electron acceptor (Fig. 5) and in the presence of CO, using mitochondrial membrane fragments sonicated for 90 seconds as a source of cytochrome P-450 (*cf.* Methods), rapid but partial reduction of cytochrome P-450 was observed with the complete system (Fig. 5A). The subsequent addition of sodium dithionite gave 100% reduction. In the absence of adrenodoxin reductase virtually no reduc-

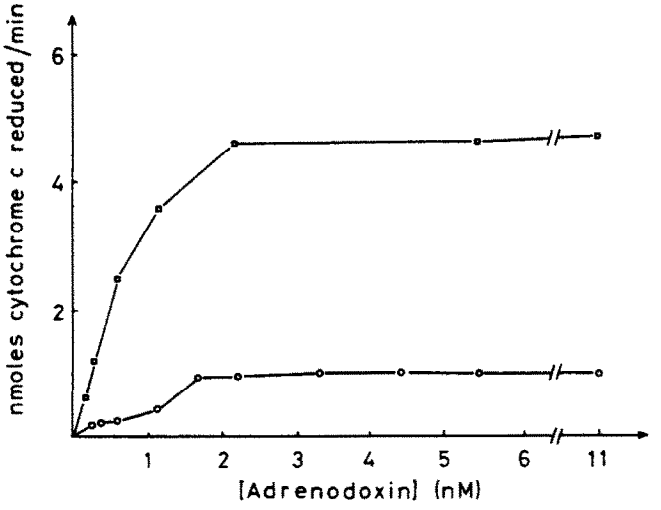

Fig. 4. Reduction of cytochrome *c* by NADPH in a reconstituted system composed of varying amounts of adrenodoxin reductase and adrenodoxin. The concentrations of adrenodoxin reductase were: (○——○), 0.36 nM; (□——□), 1.38 nM.

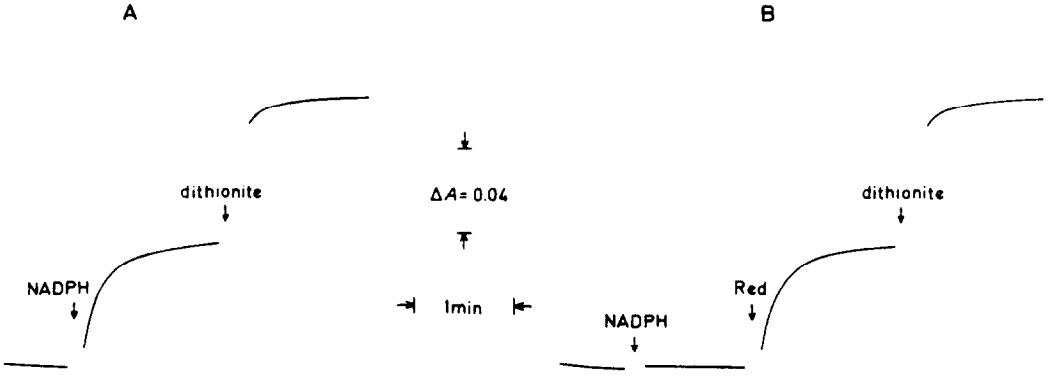

Fig. 5. Reduction of cytochrome P-450 by NADPH in a reconstituted system in the presence (A) and in the absence (B) of purified adrenodoxin reductase. (A), the reconstituted system was composed of 50 mM Tris–HCl (pH 7.0), 15 mM potassium chloride, 2 mM EDTA, 5 mM $MgCl_2$, 5 mM potassium phosphate, 20 μg adrenodoxin, 3.5 μg adrenodoxin reductase, 1.4 mg mitochondrial membrane fraction (cytochrome P-450), 2 mM isocitrate and 30 μg isocitrate dehydrogenase in a final volume of 1 ml; (B), as in (A) except that adrenodoxin reductase was omitted. The mixture was bubbled with carbon monoxide for 45 seconds and the reaction was started by the addition of 100 μM NADPH. Additions were: a few grains of sodium dithionite and 3.5 μg adrenodoxin reductase. The reaction was monitored at 450–490 nm. Temperature was 30°C.

tion of cytochrome P-450 occurred (Fig. 5B), whereas the addition of adrenodoxin reductase restored reduction of cytochrome P-450 (Fig. 5B).

Steroid hydroxylation was tested with the reconstituted system, using the mitochondrial membrane fragments described in the experiment of Fig. 5. The spectrophotometric assay of 11β-hydroxylase activity in the complete system is shown in Fig. 6A where the cyclic absorption change indicated binding of deoxycorticosterone to cytochrome $P\text{-}450_{11\beta}$ and the subsequent removal of deoxycorticosterone due to 11β-hydroxylation to corticosterone [27]. When adrendoxin reductase was omitted, only the initial phase of the cycle was observed indicating that deoxycorticosterone bound to cytochrome $P\text{-}450_{11\beta}$ but was not hydroxylated (Fig. 6B). The subsequent addition of adrenodoxin reductase gave an increase in absorbance due to the restoration of 11β-hydroxylase activity. Compared to the 11β-hydroxylase activity of intact bovine adrenal cortex mitochondria, which is of the order of 5 nmol/min/mg protein, the activity of the reconstituted system in Fig. 6B was considerably lower, or about 0.1 nmol/min/mg protein. A complete reconstituted system using mitochondrial membrane fragments, obtained by treatment with a French Press, gave an intermediate value of about 2 nmol/min/mg protein. The different 11β-hydroxylase activities are summarized in Table 2. Since disruption of mitochondial membranes by rapid decompression usually is considered to be a milder treatment than sonication, it was of interest to investigate the possibility that sonication inhibited the 11β-hydroxylase activity of the fragments. Indeed, as shown in Table 2, sonication of mitochondria for 15 min rendered the fragments essentially inactive with respect to hydroxylation of deoxycorticosterone. Similar results were obtained in experiments where conversion of radioactive dexoycorticosterone was estimated directly (not shown). However, cytochrome P-450 *per se* remained intact as judged from cytochrome P-450

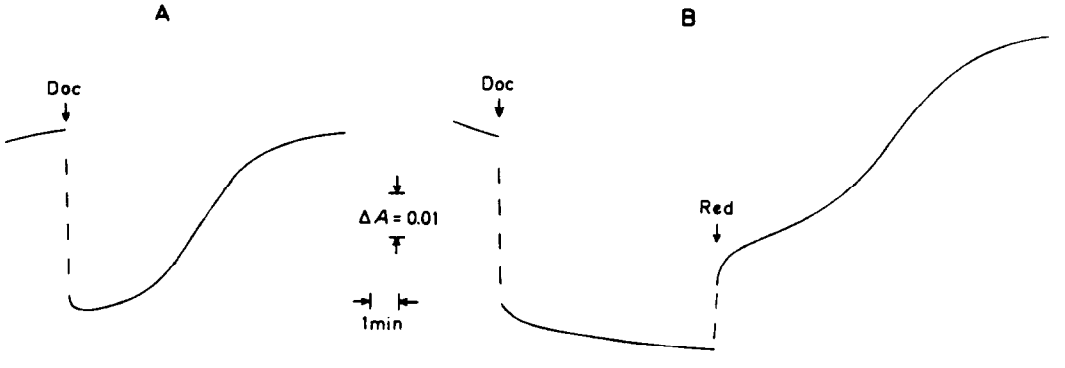

Fig. 6. Hydroxylation of deoxycorticosterone by NADPH in a reconstituted system in the presence (A) and in the absence (B) of adrenodoxin reductase. The conditions were as described in Fig. 5 except that carbon monoxide was omitted. Hydroxylation was followed at 420–390 nm. Additions were: 0.5 μg deoxycorticosterone and 3.5 μg adrenodoxin reductase.

Table 2. Cytochrome P-450 content and cytochrome P-450-linked activities in intact mitochondria and in mitochondrial membrane fragments from bovine adrenal cortex

Preparation	11β-hydroxylase activity (nmol/min/mg prot.)	Cytochrome P-450 reduction* (%)	Cytochrome P-450 content (nmol/mg prot.)
Intact mitochondira	5	30	0.5
Membrane fragments (French Press)	2	38	0.5
Membrane fragments (sonication, 90 s)	0.1	48	0.6
Membrane fragments (sonication, 15 min)	0	55	0.5

* 100% reduction was obtained with sodium dithionite.

11β-Hydroxylase catalyzed by intact mitochondria was assayed as described in Methods with 6 mM malate as reducing agent; reduction of cytochrome P-450 was carried out as shown in the legend of Fig. 5. With mitochondrial membranes these activities were assayed after reconstitution with adrenodoxin reductase and adrenodoxin as described in Fig. 5.

content per mg protein and reduction levels in complete reconstituted systems (Table 2). Also, binding of deoxycorticosterone to cytochrome P-450 was not altered significantly (not shown, *cf.* Fig. 6). These findings indicate that in the sonicated mitochondrial membrane fragments cytochrome P-450 was capable of interacting with added adrenodoxin reductase and adrenodoxin as well as with deoxycorticosterone, but was incapable of carrying out hydroxylation of deoxycorticosterone. A lack of stimulation of 11β-hydroxylase activity by the addition of supernatant obtained after sonication and centrifugation to the complete reconstituted system, indicated that no essential unknown component was solubilized during sonication.

DISCUSSION

The present communication describes a simplified procedure for the purification of homogenous adrenodoxin reductase by affinity chromatography with 2',5'-Sepharose CL-4B, which can easily be carried out in large scale. Application of the general ligand affinity chromatography technique is possible because adrenodoxin reductase is NADPH specific [29] and bound the immobilized NAPD(H) analogue 2',5'-ADP. Specific release of the bound enzyme was accomplished by free NADP(H), 2',5'ADP or 2'-AMP. Apparently, adrenodoxin reductase contaminated by adrenodoxin had a lower affinity for the column, suggesting that the binding properties of the reductase-adrenodoxin complex were markedly different as compared to those of the free enzyme. Reduction of the adrenodoxin reductase with dithionite did not increase the affinity for the immobilized nucleotide. The molecular properties of the isolated reductase investigated so far are in agreement with those described earlier [3–7, 10]. Thus, adrenodoxin reductase-catalyzed reduction of cytochrome *c* by NADPH was strictly adrenodoxin dependent and the enzyme was active in supporting electron transfer from NADPH to cytochrome P-450 as judged by cytochrome P-450 reduction and steroid hydroxylation in a system reconstituted from adrenodoxin, adrenodoxin reductase and membranes containing cytochromes P-450.

2',5'-ADP-Sepharose 4B has previously been used for the purification of NADPH-linked cytochrome *c* reductases from both rat and pig liver microsomes [30], as well as several other NADPH-linked enzymes [28]. The former preparations were obtained by the use of detergents. However, detergent-solubilized adrenodoxin reductase from adrenal cortex mitochondria bound and was eluted specifically from the affinity column, but was about one order of magnitude less active in reducing cytochrome *c* by NADPH in the presence of adrenodoxin. This preparation was less active in 11β-hydroxylation of steroids (J. Montelius and J. Rydström, unpublished). During the completion of the present work Hiwatashi *et al.*[31] described purification of adrenodoxin reductase from pig adrenal cortex mitochondria with 2',5'-ADP-Sepharose CL-4B. However, it was not shown whether the preparation was reconstitutively active with adrenodoxin and cytochrome P-450.

Successful reconstitution of 11β-hydroxylase or the cholesterol side-chain cleavage system from purified components in the absence of added lipids has been reported by several different groups [6, 13, 14, 29]. Hall and coworkers [21] showed that delipidated, inactive, cytochrome $P\text{-}450_{scc}$ was activated by endogenous phospholipids but not by phosphatidylcholine or phosphatidylethanolamine. The extent of activation of the lipid-depleted enxyme was about 1%, and the phospholipid/protein ratio used during reconstitution was below 1, indicating that the activation was due to a direct effect of catalytic amounts of phospholipid rather than to the formation of liposomes. However, a successful reconstitution of the complete cytochrome $P\text{-}450_{scc}$ system was reported recently by Hall *et al.*[22]. Takikawa *et al.*[32] demonstrated a pronounced activation of cytochrome $P\text{-}450_{scc}$ by the detergent Tween 20, similar to that observed upon reconstitution of cytochrome $P\text{-}450_{scc}$ in liposomes [22]. Therefore, it appears that an increased solubility of cholesterol, rather than a confor-

mational change of cytochrome P-450$_{scc}$, may explain this activation.

To date attempts to reconstitute the 11β-hydroxylase system in liposomes have failed. In the present investigation mitochondrial membranes devoid of adrenodoxin reductase and adrenodoxin were used as a source of cytochrome P-450$_{11\beta}$. Reconstitution of these membranes with adrenodoxin reductase and adrenodoxin yielded a system that contained cytochrome P-450 at a concentration similar to that in intact mitochondria and that catalyzed reduction of cytochrome P-450 by NADPH. In contrast, essentially no hydroxylase activity was observed with membranes exposed to prolonged sonication. A lack of transport of the steroid to the active site of cytochrome P-450$_{11\beta}$, or a modification of a part of cytochrome P-450$_{11\beta}$ required for hydroxylation, might explain this finding. However, these possibilities appear somewhat less likely since binding of the substrate to cytochrome P-450$_{11\beta}$, and therefore also transport, readily took place (*cf.* Fig. 6B). A third possibility is that increasing exposure of the membranes to sonication affected transport of electrons from adrenodoxin to cytochrome P-450$_{scc}$ and cytochrome P-450$_{11\beta}$ differently. Prolonged sonication could have exposed cytochrome P-450$_{scc}$ and shielded cytochrome P-450$_{11\beta}$ completely without any larger change in reduction of cytochrome P-450. However, a shielding of cytochrome P-450$_{11\beta}$ was ruled out by the lack of effect of added lysolecithin which disperses the membrane without inhibiting the 11β-hydroxylase activity [18]. A fourth possibility that can not be excluded at the present time is that hydroxylation of deoxycorticosterone requires an additional unknown factor which was inactivated during prolonged sonication. A hypothetical labile factor, responsible for the cycloheximide-sensitive regulation of cholesterol side-chain cleavage by adrenocorticotrophine (ACTH) was recently proposed by Simpson *et al.*[33, 34]. This factor was suggested to increase the interaction between the cytochrome P-450$_{scc}$ and cholesterol, an effect which was dependent of the intactness of the mitochondria. Even though hydroxylation of deoxycorticosterone does not seem to be affected by ACTH [33, 35] the possible existence of a similar factor involved in 11β-hydroxylation has to be considered. This possibility is presently being investigated.

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