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Mechanisms of Ionic Activation of Adrenal Mitochondrial Cytochromes P-450$_{scc}$ and P-450$_{11\beta}$*

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The effects of metal ions on the enzymatic reduction of adrenodoxin, on cholesterol side chain cleavage, and on 11β-hydroxylation, all catalyzed by purified enzymes, have been compared. Both monovalent and divalent ions activated adrenodoxin reduction by NADPH and adrenodoxin reductase, confirming previous findings (Lambeth, J. D., Seybert, D. W., and Kamin, H. (1979) *J. Biol. Chem.* 254, 7255-7264). Increasing ionic strength continuously increased the V_{max} for cholesterol side chain cleavage when adrenodoxin was both saturating and fully reduced by excess adrenodoxin reductase. No effect of ionic strength on 11β-hydroxylase V_{max} was observed under these conditions. At lower [adrenodoxin], both activities declined at high ionic strength due to similar increases in K_m for adrenodoxin. The decreases in activity were well described by a simple Michaelis-Menten function of V_{max}, K_m and [adrenodoxin$_{free}$]. When [adrenodoxin reductase] was insufficient to fully reduce adrenodoxin, inhibition of both side chain cleavage and 11β-hydroxylation by oxidized adrenodoxin was demonstrable. For 11β-hydroxylation, a decrease in ion concentration (80 to 40 mM NaCl; 5 to 1 mM $CaCl_2$) greatly decreased inhibition by oxidized adrenodoxin (full activity with 40% oxidized adrenodoxin), while for side chain cleavage, this inhibition was marked under all conditions. 11β-Hydroxylation was activated to the same maximum activity (70 min^{-1} at 30 °C) by $CaCl_2$ (2 mM) and NaCl (100 mM). In contrast, side chain cleavage activation by $CaCl_2$ reached at most only 15% of that by NaCl. $CaCl_2$ (30 to 100 μM) strongly inhibited side chain cleavage after activation by 100 mM NaCl. This inhibition was associated with decreased electron transfer from reduced adrenodoxin to P-450$_{scc}$ under steady state conditions. Magnesium chloride fully stimulated both monooxygenases.

In the adrenal cortex, three steps of steroidogenesis are catalyzed by mitochondrial cytochromes P-450: cholesterol side chain cleavage, and 11β- and 18-hydroxylation of Δ^4-3-ketosteroids (1-3). While cytochrome P-450$_{scc}$[1] specific for cholesterol side chain cleavage is distinct from cytochrome P-450$_{11\beta}$ specific for 11β-hydroxylation (2, 3), the latter can also catalyze 18-hydroxylation of 11-deoxycorticosteroids (4, 5). The mitochondrial cytochromes P-450 are dependent on a ferredoxin-type iron sulfur protein and a flavoprotein with one cofactor (FAD) which acts as the ferredoxin reductase (6-8). In adrenal cortex mitochondria, the concentration of P-450 is equal to that of adrenal ferredoxin (adrenodoxin), while the concentration of the flavoprotein adrenodoxin reductase appears to be one-tenth of these enzymes (9, 10). The mitochondrial cytochromes P-450, like all cytochrome P-450 isolated from mammalian tissues, are integral membrane proteins, while in contrast, adrenodoxin and adrenodoxin reductase behave as peripheral membrane proteins (2, 3, 11-13).

Recent studies with purified enzymes indicate that adrenodoxin transfers electrons from adrenodoxin reductase to P-450 by shuttling between these two enzymes and not within a ternary complex of the three proteins (12-14). Metal ions strongly modulate adrenodoxin binding to adrenodoxin reductase and to P-450 and can activate adrenodoxin reduction by adrenodoxin reductase, as well as cholesterol side chain cleavage and 11β-hydroxylation (13, 16-19). The mechanism of ionic activation of adrenodoxin reduction by adrenodoxin reductase has been elucidated by the recent studies of Lambeth *et al.* (18). In a preliminary communication of the results presented here, we have noted that the ionic activation of the monooxygenase activities of cytochromes P-450$_{scc}$ and P-450$_{11\beta}$ is the combined result of several different processes and cannot be explained solely by the activation of adrenodoxin reduction (20).

In the present studies with purified adrenodoxin, adrenodoxin reductase, P-450$_{scc}$, and P-450$_{11\beta}$, we have examined the effect of univalent and bivalent metal ions on the reduction of adrenodoxin reductase and on the side chain cleavage and 11β-hydroxylation activities of the cytochromes, all under the same conditions. Our results indicate that ions, particularly Ca^{2+}, affect side chain cleavage and 11β-hydroxylation differently. Previously, we have provided evidence that oxidized adrenodoxin (ADX^o) inhibits side chain cleavage activity by competing with reduced adrenodoxin (ADX^r) for binding to P-450$_{scc}$ (15). The present results indicate that the same effect also operates with P-450$_{11\beta}$ and that ionic activation of 11β-hydroxylation appears to be associated with a decreased effectiveness of this competition.[2]

EXPERIMENTAL PROCEDURES

Materials—The phospholipids were purchased from Serdary and Tween 20 and Hepes from Sigma Chemical Co. The other materials

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[1] The abbreviations and trivial names used are: P-450$_{scc}$, cytochrome P-450 specific for cholesterol side chain cleavage; P-450$_{11\beta}$, cytochrome P-450 specific for 11β-hydroxylation of Δ^4-3-ketosteroids; ADX^o, oxidized form of adrenodoxin; ADX^r, reduced form of adrenodoxin; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; Hepes, 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid.

[2] These studies were presented in part at the American Society of Biological Chemists Minisymposium on Cytochrome P-450, New Orleans, LA, June 6, 1980.

were obtained from previously indicated sources (21). [1α-2α(2n)-^{3}H]-Cholesterol (43 Ci/mmol) was purified as previously described (21).

Preparation of Phospholipid Vesicles—Unilamellar cholesterol-containing phospholipid vesicles was prepared according to the procedure of Lambeth *et al.* (18) with some modifications. Aliquots of nonradioactive cholesterol and [^{3}H]cholesterol solutions in ethanol and benzene were placed in a 40-ml conical Pyrex tube to give a final specific activity of 6.3 cpm/pmol. The solvents were evaporated and the phospholipid added in chloroform and dried under N_2. After completely drying the phospholipid, the buffer (20 mM Hepes, pH 7.2, with or without 100 mM NaCl) was added and the solution sonicated for 40 min under a gentle stream of N_2 with the tube immersed in a circulating water bath at room temperature. The concentrations of the phospholipid and cholesterol in the sonicated solution were always 1 mg/ml of buffer and 600 μM, respectively. The volume of the sonicated solution varied between 3 to 5 ml. The vesicles were used the same day and kept under a N_2 atmosphere until use in assays.

Enzyme Purifications—Adrenodoxin and cytochrome P-450$_{scc}$ were purified as previously described (13, 15). Adrenodoxin reductase purification was carried out according to the procedure of Hiwatashi *et al.* up to the second DEAE-cellulose chromatography step (22). Approximately 130 nmol of the ammonium sulfate concentrated and dialyzed reductase preparation was applied on an adrenodoxin-Sepharose column (0.9 × 8 cm) equilibrated with 10 mM K phosphate buffer (KP), pH 7.4. The column was washed with 15 mM KP, pH 7.4, and then the reductase was eluted with 50 mM KP, 0.5 M KCl, pH 7.3. The fractions with A_{272}/A_{450} = 8.2 or less were combined, dialyzed against 50 mM KP, pH 7.4, for 4 h and stored in 100-μl portions at −80 °C. Cytochrome P-450$_{11\beta}$ was purified to A_{280}/A_{390} = 0.8 to 0.9, and 15 to 16 nmol/mg of protein by a modification of the procedure of Suhara *et al.* (23) in which adrenodoxin-Sepharose chromatography was added as a final step. The enzyme was dialyzed against 25 mM K phosphate (pH 7.3) containing 50 μM EDTA, 50 μM dithiothreitol, 25 μM deoxycorticosterone, 0.25% Tween 20, and 0.25% sodium cholate, and it was added to assays in this medium.

Assays—The assays involving the measurement of both adrenodoxin reduction and side chain cleavage activity were carried out in 10 mM Hepes (pH 7.2) containing 0.3% Tween 20 and 200 μM [^{3}H]-cholesterol in a final volume of 1 ml in thermostated cuvettes in an Aminco DW-2 spectrophotometer. After monitoring the reduction of adrenodoxin spectrophotometrically, 200-μl aliquots of the reaction solution were removed at desired times and placed in 200 μl of ethanol in 5-ml polypropylene tube. The steroids were then extracted with methylene chloride and the product, [^{3}H]pregnenolone, was separated from the substrate and quantitated as previously described (21).

The assay of side chain cleavage activity of phospholipid vesicle reconstituted P-450$_{scc}$ was carried out in 20 mM Hepes (pH 7.2) containing 600 μM dioleoylphosphatidylcholine (in the form of unilamellar cholesterol containing phospholipid vesicles prepared as described above) and 300 μM [^{3}H]cholesterol in a final volume of 200 μl using procedures similar to those previously described (21). The major difference was that P-450$_{scc}$ was added to the reaction mixture, lacking only the enzymes and NADPH, mixed by Vortex and preincubated at 37 °C for 3 min; adrenodoxin, adrenodoxin reductase, and NADPH (1 mM) were then added in that order to start the reaction. At the stage of steroid extraction, after removal of the aqueous layer, the methylene chloride layer was re-extracted with 0.2 ml of H_2O.

The assays involving the measurement of both adrenodoxin reduction and 11β-hydroxylase activity were carried out in 10 mM Hepes (pH 7.2) containing 50 μM deoxycorticosterone (stock solution, 20 mM in ethanol) in a final volume of 1 ml in thermostated cuvettes essentially as described for side chain cleavage above. The final concentration of cholate and Tween 20 added with the cytochrome was 0.01%. P-450$_{11\beta}$ is relatively unstable and was added immediately prior to initiation of the reaction with NADPH. After the reactions were stopped with 200 μl of ethanol, the steroids were extracted with 2 ml of methylene chloride, and the product, corticosterone, was assayed fluorimetrically (24).

The side chain cleavage reactions were carried out for 4 min at 37 °C and the 11β-hydroxylation reactions for 1 or 2 min at 30 °C. The formation of products increased linearly within these time periods. The percentage of adrenodoxin in the reduced state was determined as previously described (15).

Data Analysis and Simulations—The concentrations of adrenodoxin$_{free}$ were calculated as described previously (15). The K_d for the adrenodoxin reductase-adrenodoxin complex at each ion concentration was taken from values determined by Lambeth *et al.* (18). For Fig. 8, the K_d values for binding of adrenodoxin to P-450$_{11\beta}$ were taken to be the same as previously determined for P-450$_{scc}$ at these ion concentrations (13). For Fig. 9, the K_d for the adrenodoxin-P-450$_{11\beta}$ complex was taken as 0.2×10^{-7} M which provided the best fit for the data. The lines in Figs. 8 and 9 are based on nonlinear regression analysis using the Fortran program HYPER (25).

The simulated curves in Fig. 2 were generated using Equation 1 under "Results." This required the definition of V_{max} for cholesterol side chain cleavage, K_m for adrenodoxin in side chain cleavage, and K_d values for adrenodoxin complex formation with adrenodoxin reductase and P-450$_{scc}$ (for use in the calculation of adrenodoxin$_{free}$) as functions of ionic strength (I). The function taken for V_{max} was the linear regression line fitted to the data points from 50 to 200 mM NaCl (Fig. 2). The K_m for adrenodoxin and the K_d for adrenodoxin complex formation with adrenodoxin reductase and P-450$_{scc}$ were defined as functions of I, in terms of their experimentally determined values up to 200 mM NaCl (13, 18). Values at higher I were difficult to obtain experimentally and were therefore determined by extrapolation of the data up to 200 mM NaCl.

RESULTS

Effects of Ions on Cholesterol Side Chain Cleavage and Adrenodoxin Reduction—Cholesterol side chain cleavage reconstituted with purified adrenodoxin, adrenodoxin reductase, and P-450$_{scc}$ showed a similar bell-shaped dependence on NaCl both in Tween 20 and phospholipid vesicles (Fig. 1). The dependence on KCl in Tween 20 was essentially identical with NaCl (data not shown and Ref. 19). In the absence of NaCl, side chain cleavage activity was undetectable, while under the same conditions, about 60% of the adrenodoxin molecules were in the reduced form (Fig. 1). As [NaCl] was increased, the steady state levels of ADX^r and side chain cleavage activity increased nearly in parallel until all adrenodoxin molecules became reduced at 50 to 60 mM NaCl. Side chain cleavage activity continued to increase from 50 to 100 mM NaCl, then decreased as [NaCl] was increased further, whereas all adrenodoxin molecules remained reduced even at 300 mM NaCl (Fig. 1).

We have recently noted that ADX^o inhibits side chain

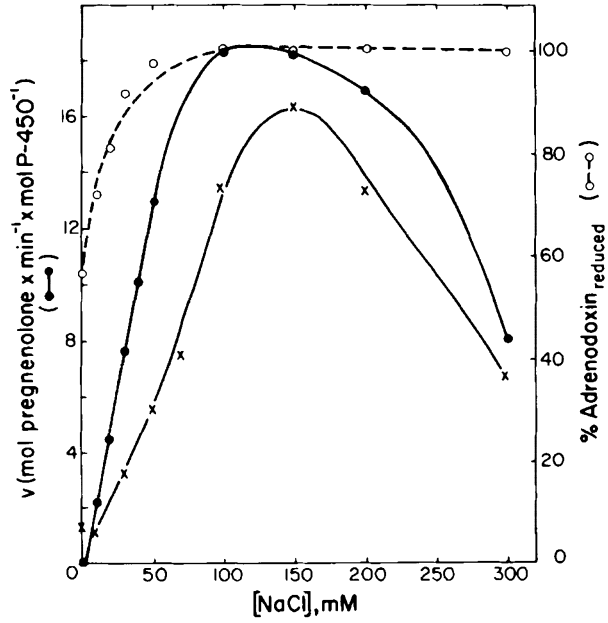

FIG. 1. **Effect of NaCl on adrenodoxin reduction and cholesterol side chain cleavage activity in Tween 20 (O- - -O, ●——●) or phospholipid vesicles (×——×).** The assays were carried out in 10 mM Hepes (pH 7.2) with 0.3% Tween 20, 200 μM cholesterol, 0.12 μM adrenodoxin reductase, 7.5 μM adrenodoxin, and 0.2 μM P-450$_{scc}$; or in 20 mM Hepes (pH 7.2) with 600 μM dioleoylphosphatidylcholine, 300 μM cholesterol, 0.24 μM adrenodoxin reductase, 7.5 μM adrenodoxin, and 0.28 μM P-450$_{scc}$.

cleavage probably by competing with ADX^r for binding to $P\text{-}450_{scc}$ (15) and that the V_{max} for side chain cleavage at saturation with adrenodoxin increases linearly with ionic strength from 50 to 200 mM NaCl (13). Therefore, the steep increase of side chain cleavage observed from 0 to 50 mM NaCl in Fig. 1 could be ascribed to a decrease in [ADX^o] and/or an increase in the rate of side chain cleavage independent of changes in the steady state [ADX^o]. In order to distinguish between these possibilities, the experiments in Fig. 2 were conducted with a concentration of adrenodoxin reductase (4 to 5 times higher than in Fig. 1) which was sufficient to reduce all adrenodoxin molecules even at 0 mM NaCl. Under these conditions, side chain cleavage activity increased sharply with increased [NaCl]. However, from 0 to about 50 mM NaCl with saturating [ADX^r], the turnover numbers in Fig. 2 were significantly higher than those in Fig. 1, presumably due to the inhibitory effect of ADX^o under the conditions used in Fig. 1. We have recently observed that the K_m for adrenodoxin in cholesterol side chain cleavage increases exponentially with I so that [adrenodoxin] becomes limiting at high I (13). The side chain cleavage activities shown at 100 and 200 mM NaCl in Fig. 2 represent the V_{max} at saturation with adrenodoxin, determined with extrapolation of v^{-1} *versus* $[\text{adrenodoxin}]^{-1}$ (13).

The simulated curves in Fig. 2 show that the decline in side chain cleavage activity at high I can be well accounted for by the exponential increase in the K_m for adrenodoxin. These simulations are based on the Michaelis-Menten Equation 1 below:

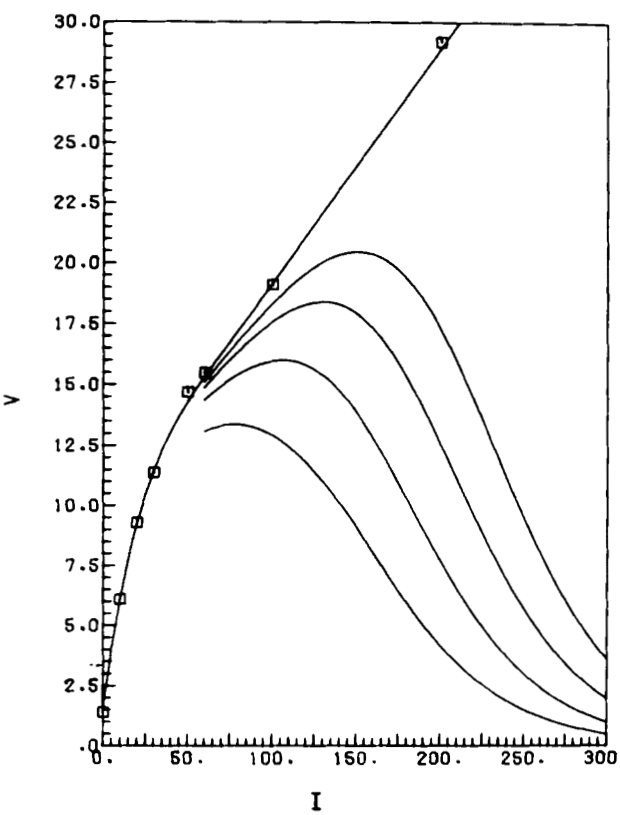

FIG. 2. **Effect of NaCl on cholesterol side chain cleavage in the presence of fully reduced adrenodoxin in Tween 20.** The assay conditions were as in Fig. 1 except that [adrenodoxin reductase] was 0.6 μM at 0 mM NaCl and 0.48 μM at 10 to 60 mM NaCl. The values for 100 and 200 mM NaCl represent extrapolated V_{max} values at saturation with adrenodoxin. The top bell-shaped curve was generated using Equation 1 under "Results" with the experimental conditions of Fig. 1. The curves below that were generated for the same conditions except assuming 4.0, 2.0, or 1.0 μM adrenodoxin concentration, respectively, for each of the curves.

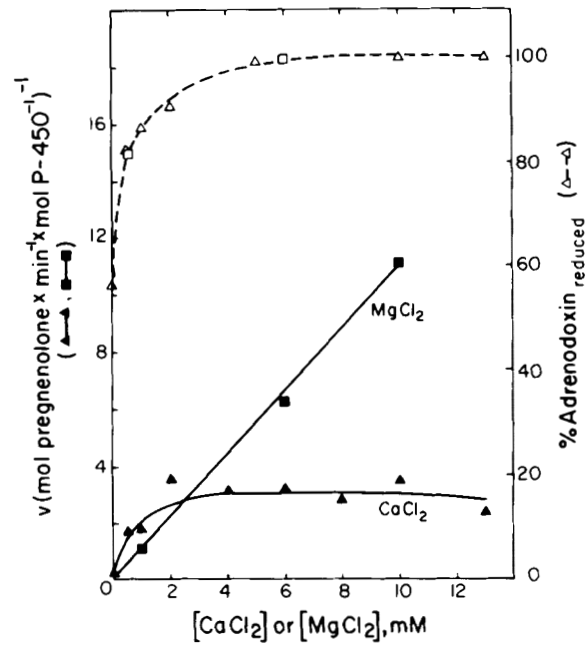

FIG. 3. **Effect of $CaCl_2$ and $MgCl_2$ on adrenodoxin reduction and cholesterol side chain cleavage activity in Tween 20.** The assay conditions were as in Fig. 1.

$$v = \frac{V_{max} \cdot [ADX_{free}]}{K_m + [ADX_{free}]} \quad (1)$$

in which V_{max} and K_m represent values at each I and $\text{adrenodoxin}_{free}$ is calculated taking into account the K_d values for complex formation with adrenodoxin reductase and $P\text{-}450_{scc}$ at each I. A noteworthy point in these simulations is that as [adrenodoxin] is lowered, the peaks of the curves are shifted to lower I and the peaks become narrower. Indeed, both we (data not shown) and Takikawa *et al.* (19) have observed sharper peaks with lower [adrenodoxin]. Although the generated patterns are similar to those found experimentally (Fig. 1) with 7.5 and 4.0 μM adrenodoxin, we have consistently observed higher turnover numbers at 300 mM NaCl than those predicted by the simulations. An error in K_m is the likely source of this discrepancy. An estimated K_m for adrenodoxin (40 μM) at 300 mM NaCl was obtained by extrapolation of the linear plot of log K_m *versus* I (50 to 200 mM NaCl; Ref. 13, Fig. 8). This K_m could easily exceed the true value by a factor of 2, particularly in view of the observed saturation of the analogous plot of log K_d *versus* I (13).

In contrast to univalent metal ions, Ca^{2+} was a very ineffective activator of side chain cleavage activity reconstituted in Tween 20 (Fig. 3) and caused no significant activation of side chain cleavage reconstituted with phospholipid vesicles. The low activation could not be ascribed to the presence of ADX^o because at 3 to 4 mM $CaCl_2$ all adrenodoxin molecules were reduced (Fig. 3). The concentration range of $MgCl_2$ necessary to increase the steady state levels of ADX^r was similar to $CaCl_2$ (Fig. 3). However, in contrast to $CaCl_2$, increased [$MgCl_2$] continued to increase side chain cleavage activity even after all adrenodoxin molecules were reduced (Fig. 3). A replot of $MgCl_2$ activation of side chain cleavage activity on the basis of ionic strength was nearly superimposable on that for NaCl.

In the presence of 100 mM NaCl, $CaCl_2$ inhibited the side chain cleavage activity of $P\text{-}450_{scc}$ in both Tween 20 micelles and phospholipid vesicles (Fig. 4). $MgCl_2$ caused no inhibition under these conditions. The [Ca^{2+}] necessary for 50% inhibition of side chain cleavage activity was 70 μM in Tween 20 and

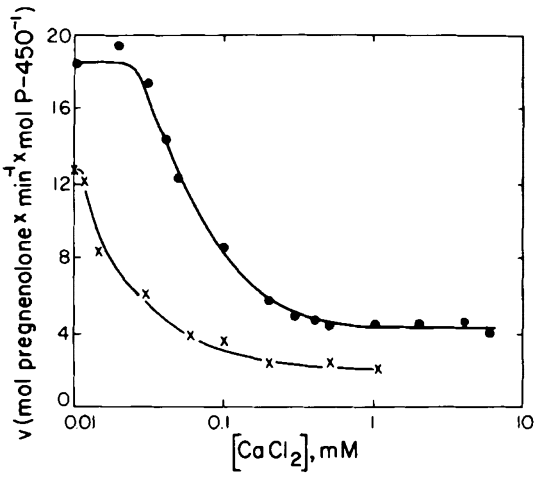

FIG. 4. **Effect of $CaCl_2$ on cholesterol side chain cleavage activity in Tween 20 (●——●) and phospholipid vesicles (×——×) in the presence of 100 mM NaCl.** The assays were carried out in 10 mM Hepes (pH 7.2) with 0.3% Tween 20, 200 μM cholesterol, 0.12 μM adrenodoxin reductase, 7.5 μM adrenodoxin, 0.16 μM $P\text{-}450_{scc}$, and 100 mM NaCl; or in 20 mM Hepes (pH 7.2) with 600 μM dioleoylphosphatidylcholine (sonicated with cholesterol, and 100 mM NaCl), 300 μM cholesterol, 0.24 μM adrenodoxin reductase, 7.5 μM adrenodoxin, 0.28 μM $P\text{-}450_{scc}$, and 100 mM NaCl.

30 μM for vesicle-incorporated P-450 (Fig. 4). This inhibition could not be due to Ca^{2+} effects on adrenodoxin reduction because, in the presence of 100 mM NaCl, Ca^{2+} had no significant effect on either the rate of adrenodoxin reduction by adrenodoxin reductase or the steady state levels of ADX^r. The effect of Ca^{2+} on electron transfer from adrenodoxin to P-450 was analyzed by examining the effect on the steady state $[ADX^r]$ during cholesterol side chain cleavage. In absence of $P\text{-}450_{scc}$, 17 nM adrenodoxin reductase was sufficient to nearly fully reduce adrenodoxin (Fig. 5). Addition of $P\text{-}450_{scc}$ resulted in a new steady state in which only 60% of adrenodoxin molecules became reduced (Fig. 5). Addition of Ca^{2+} during side chain cleavage increased the steady state $[ADX^r]$ indicating that Ca^{2+} inhibition of side chain cleavage reaction was associated with a decreased rate of oxidation of ADX^r. The effect of Ca^{2+} could be reversed by nearly stoichiometric concentrations of EDTA (Fig. 5) which at these concentrations, in absence of Ca^{2+}, did not affect the side chain cleavage activity or the steady state $[ADX^r]$ during side chain cleavage. However, addition of increasing concentrations of EDTA in the presence of Ca^{2+} resulted in a further decrease of the proportion of ADX^r below that observed in absence of Ca^{2+}. At present, the reason for this effect is not clear.

Effects of Ions on 11β-Hydroxylation and Adrenodoxin Reduction—In contrast to $P\text{-}450_{scc}$, the catalytic activity of $P\text{-}450_{11\beta}$ was activated to the same maximum activity by both monovalent (Na^+, K^+) and divalent (Ca^{2+}, Mg^{2+}) metal ions (Figs. 6 to 8). The bivalent ions stimulated at much lower ionic strength than monovalent ions. The activation of the hydroxylase activity was associated with an increase in the proportion of ADX^r at steady state (Figs. 6 and 7). However, under these conditions, 11β-hydroxylation reached maximal levels at 40 mM NaCl or 2 mM $CaCl_2$ while 30 to 40% of adrenodoxin was oxidized (Figs. 6 and 7). As ion concentrations were further increased, all adrenodoxin molecules became reduced and remained reduced even at the highest I examined (300 mM NaCl). Full reduction of adrenodoxin required higher ion concentrations than those required for side chain cleavage (Figs. 1 and 3), probably because of the higher rate of ADX^r oxidation by $P\text{-}450_{11\beta}$ and the lower rate of adrenodoxin reduction at 30 °C.

As shown in Fig. 8, the K_m for adrenodoxin increased exponentially with I in a manner similar to that observed with $P\text{-}450_{scc}$ (13). However, in contrast to $P\text{-}450_{scc}$, the V_{max} at saturation with adrenodoxin remained essentially constant with ionic strength up to 200 mM KCl (Fig. 8). Thus, the decline in 11β-hydroxylase activity seen at high I in the presence of constant $[ADX^r]$ (Fig. 6) was also due to the increase in the K_m for ADX. The V_{max} observed in 5 mM $CaCl_2$

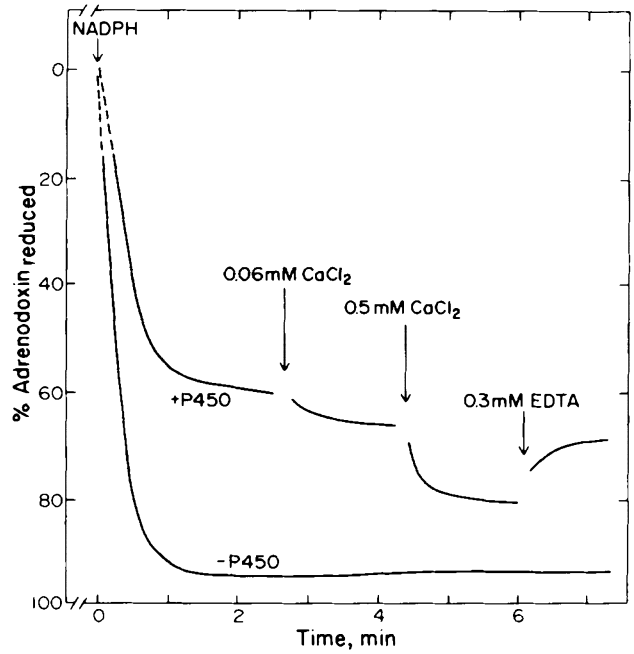

FIG. 5. **Effect of $CaCl_2$ on steady state levels of reduced adrenodoxin during cholesterol side chain cleavage in Tween 20.** The figure shows the actual recorder tracings of absorbance change at 456 nm. The *dotted lines* represent extrapolations to zero time during the initial mixing phase. The assays were carried out in 10 mM Hepes (pH 7.2) containing 0.3% Tween 20, 200 μM cholesterol, 100 mM NaCl, 17 nM adrenodoxin reductase, 7.9 μM adrenodoxin, with (+P-450) or without (−P-450) 0.19 μM $P\text{-}450_{scc}$.

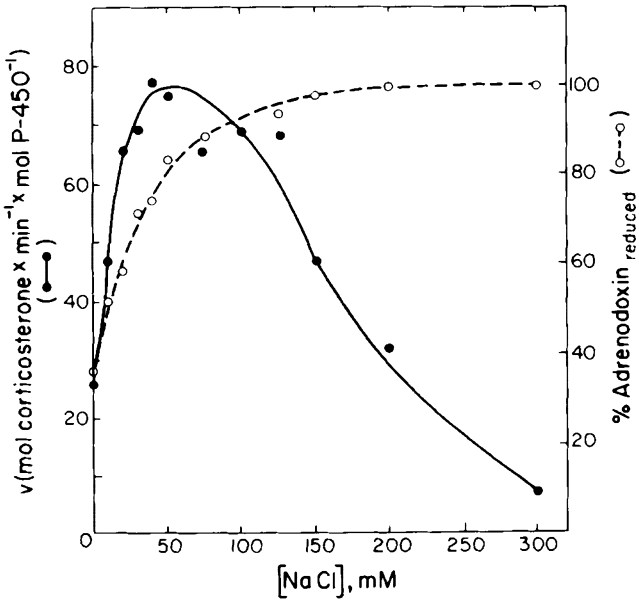

FIG. 6. **Effect of NaCl on adrenodoxin reduction and 11β-hydroxylase activity.** The assays were carried out in 10 mM Hepes (pH 7.2) with 50 μM deoxycorticosterone, 0.12 adrenodoxin reductase, 7.2 μM adrenodoxin, and 0.165 μM $P\text{-}450_{11\beta}$.

was not different from that observed with up to 150 mM KCl (Fig. 9). The small decline in activity seen at 10 mM $CaCl_2$ (Fig. 7) cannot be interpreted as a significant effect, because, at these concentrations, $CaCl_2$ causes much aggregation and turbidity in the reaction mixture.

Previously, we noted that ADX° inhibits side chain cleavage activity probably by competing with ADX^r for binding to P-450_{scc} (15). Therefore, the observation that 11β-hydroxylation reaches V_{max} in the presence of substantial [ADX°] (Figs. 6 and 7) was surprising. Thus, we examined whether ADX° inhibition of monooxygenase activity may be modulated by ions. We varied the steady state [ADX°] in the presence of constant total [adrenodoxin] by varying [adrenodoxin reductase] (Figs. 10 and 11). At 80 mM NaCl or 5 mM $CaCl_2$ per cent ADX^r and activity correlated directly. However, at lower ion concentrations (40 mM NaCl or 1 mM $CaCl_2$; chosen on the basis of the results in Figs. 6 and 7), activity increased more rapidly than the percentage of ADX^r. In particular,

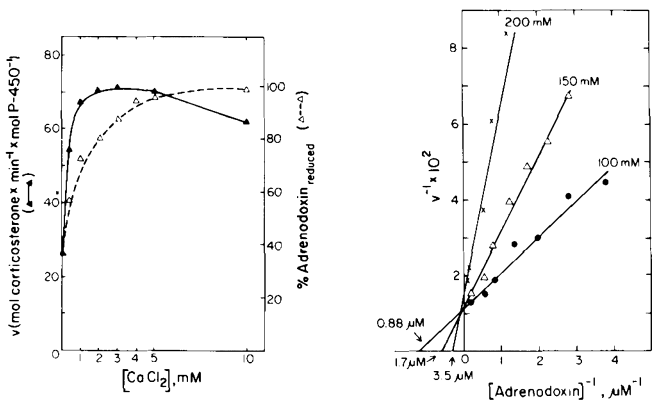

FIG. 7 (*left*). **Effect of $CaCl_2$ on adrenodoxin reduction and 11β-hydroxylase activity.** The assay conditions were as in Fig. 6.

FIG. 8 (*right*). **Effect of KCl on adrenodoxin dependence of 11β-hydroxylase activity.** The [adrenodoxin] on the abscissa refer to [adrenodoxin$_{free}$]. The assays were carried out in 10 mM Hepes (pH 7.2) with 50 μM deoxycorticosterone, 0.24 μM adrenodoxin reductase, and 0.2 μM P-$450_{11\beta}$. The concentrations of KCl are shown next to each plot. The numbers on the lower left of the figure represent the apparent K_m for adrenodoxin at each concentration of KCl.

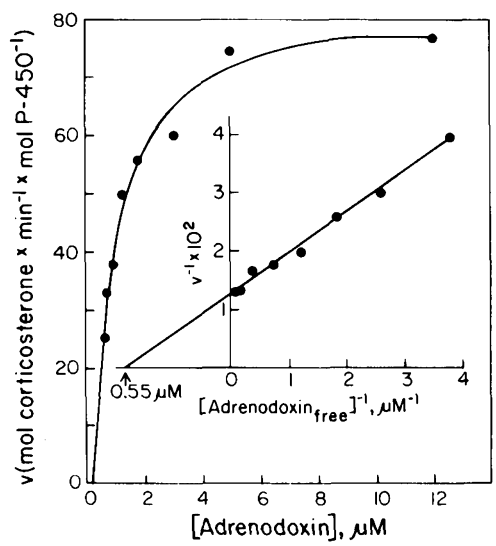

FIG. 9. **Adrenodoxin dependence of 11β-hydroxylase activity in the presence of 5 mM $CaCl_2$.** The assays were carried out in 10 mM Hepes (pH 7.2) with 50 μM deoxycorticosterone, 0.24 μM adrenodoxin reductase, and 0.165 μM P-$450_{11\beta}$. In the *inset*, the same data are replotted in double reciprocal form.

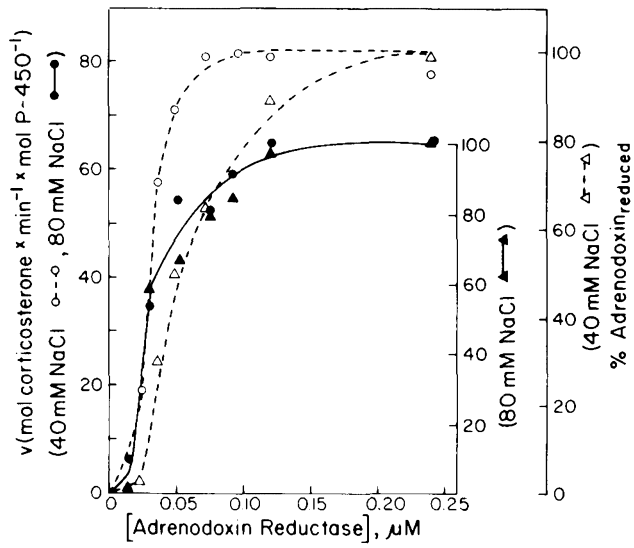

FIG. 10. **Effect of 40 mM and 80 mM NaCl on adrenodoxin reductase dependence of adrenodoxin reduction and 11β-hydroxylase activity.** The assays were carried out in 10 mM Hepes (pH 7.2) with 50 μM deoxycorticosterone, 7.6 μM adrenodoxin, and 0.15 μM P-$450_{11\beta}$.

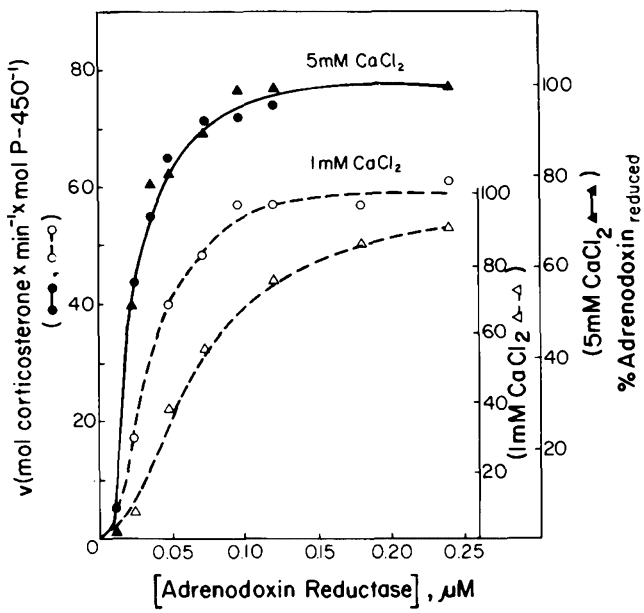

FIG. 11. **Effect of 1 mM and 5 mM $CaCl_2$ on adrenodoxin reductase dependence of adrenodoxin reduction and 11β-hydroxylase activity.** The assay conditions were as in Fig. 10.

increasing the steady state level of ADX° up to 30% did not diminish activity at the lower ion concentrations (Figs. 10 and 11).

DISCUSSION

Recent evidence indicates that adrenodoxin transports electrons from adrenodoxin reductase to P-450_{scc} or P-$450_{11\beta}$ by shuttling between these two enzymes and not within a ternary complex (12–15). As a consequence of this shuttle mechanism, cholesterol side chain cleavage activity shows a Michaelis-Menten dependence on free ADX^r (15). Electron transfer from ADX^r to P-450_{scc} during cholesterol side chain cleavage appears to be inhibited competitively by ADX° (15). The present results indicate that these principles also hold true for P-$450_{11\beta}$. Thus, the adrenodoxin dependence of the activities of

both cytochromes, at a fixed level of steroid substrate, should be described by the familiar Michaelis-Menten equation that takes into account competitive inhibition:

$$v = \frac{V_{max} \cdot [ADX^r_{free}]}{K_m \left(1 + \frac{[ADX^o_{free}]}{K_i}\right) + [ADX^r_{free}]} \quad (2)$$

in which the definition of ADX_{free} includes the K_d values for the formation of separate complexes by adrenodoxin reductase and P-450 (15).

Previous studies have shown that the K_d for cholesterol binding to $P\text{-}450_{scc}$ and the K_m for cholesterol in side chain cleavage are almost independent of ionic effects (13). However, monovalent and divalent ions can affect most if not all of the variables in Equation 2 for both cytochromes including the K_d values that determine ADX_{free}. Ions activate adrenodoxin reduction by adrenodoxin reductase, resulting in an increase in $[ADX^r]$ and a decrease in $[ADX^o]$. The K_m for ADX^r in both cholesterol side chain cleavage (13) and 11β-hydroxylation (Fig. 8), and the K_d values for complex formation with adrenodoxin reductase (18) and $P\text{-}450_{scc}$ (13) increase with ionic strength. In the special case where adrenodoxin is maintained fully reduced (Fig. 2), Equation 2 simplifies to Equation 1 which provides a good description of the effect of ionic strength on side chain cleavage activity (Fig. 2).

The effectiveness of ADX^o to inhibit monooxygenase activity also appears to be modulated by ions at least for $P\text{-}450_{11\beta}$ (Figs. 6, 7, 10, and 11). The high correlation between per cent of ADX^r and per cent maximal activity at 80 mM NaCl and 5 mM $CaCl_2$ with near saturating adrenodoxin indicates that at these ion concentrations, the K_i for ADX^o is approximately equal to the K_m for ADX^r as previously noted for $P\text{-}450_{scc}$ at an ionic strength equivalent to 80 mM NaCl (15). However, at low concentrations of both ions (40 mM NaCl, 1 mM $CaCl_2$), the observation of maximal rates of 11β-hydroxylation in the presence of substantial $[ADX^o]$ (Figs. 6, 7, 10, and 11) suggests that at these ion concentrations, ADX^o does not effectively inhibit 11β-hydroxylase activity (*i.e.* in equation 2, $K_i \gg K_m$). For $P\text{-}450_{scc}$, a comparison of the results in Figs. 1 and 2 in the range of 0 to 30 mM NaCl, where there were substantial $[ADX^o]$ in Fig. 1 and no ADX^o in Fig. 2, indicates that ADX^o inhibits side chain cleavage also at this low range of ionic strength.

In the present studies, one of the major differences observed between $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$ is that the V_{max} of side chain cleavage, but not 11β-hydroxylation, is increased by increased ionic strength independent of changes in $[ADX^r]$ (Figs. 2 and 8). At present, this activation of the rate of side chain cleavage cannot be explained at a molecular level. The studies on cytochrome $P\text{-}450_{cam}$ catalyzed-camphor monooxygenation indicate that, for this reaction, the rate-limiting step is that of the second electron transfer from putidaredoxin to $P\text{-}450_{cam}$ (26). However, we have recently discussed the possibility, based on the high ratio of K_m/K_d for adrenodoxin, that for $P\text{-}450_{scc}$, the dissociation of ADX^o from $P\text{-}450_{scc}$ may be a rate-limiting step in side chain cleavage. Although this remains speculative, it is notable that increasing ionic strength also weakens the binding of ADX^o to oxidized P-450 ($\log K_d \propto I$). Such an effect would be analogous to the ionic enhancement of adrenodoxin reduction by the reductase (18). A comparison of the data in Figs. 1 and 6 indicates that the rate of 11β-hydroxylation at a fixed [adrenodoxin] declines more rapidly at high I than does the rate of cholesterol side chain cleavage. This is consistent with activities described by Equation 2 as K_m values for both activities increase similarly with I (Fig. 8 and Ref. 13), while only V_{max} for side chain cleavage increases to partially offset this effect.

Another major difference between $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$ is that while 1 to 2 mM $CaCl_2$ activates 11β-hydroxylation to the same extent as 40 mM NaCl and KCl, it causes no significant activation of cholesterol side chain cleavage and, in the presence of 100 mM NaCl, inhibits optimally activated side chain cleavage reaction in both detergent micelles and phospholipid vesicles (Fig. 4). The inhibition of side chain cleavage is associated with a decreased rate of electron transfer from ADX^r to $P\text{-}450_{scc}$ (Fig. 5). However, this finding does not mean that Ca^{2+} is inhibiting side chain cleavage directly at this process. We have previously shown that Ca^{2+} does not affect the binding of either cholesterol or ADX^o to $P\text{-}450_{scc}$ (13). Lambeth *et al.* have recently reported that $CaCl_2$ is more effective than NaCl in the stimulation of cholesterol side chain cleavage activity of phospholipid vesicle reconstituted $P\text{-}450_{scc}$ (18). However, under the conditions of their experiments with 1 μM adrenodoxin, the turnover of P-450 was 10 to 20 times lower than that obtained in the present experiments, and the maximal activities they observed with $CaCl_2$ are lower than the maximally inhibited turnover numbers we observe. The side chain cleavage inhibitory effect of Ca^{2+} is not common to bivalent cations in general, since $MgCl_2$ causes no inhibition and activates side chain cleavage in a manner similar to NaCl on the basis of ionic strength.

At present, the physiological relevance of the opposing effects of low concentrations of Ca^{2+} on the monooxygenase enzymes is questionable. Extensive evidence indicates that Ca^{2+} plays a stimulatory role in steroidogenesis (27–32), and to our knowledge, no inhibitory effect of Ca^{2+} on NADPH-supported steroidogenesis at such low concentrations (30 to 100 μM) has been observed. In studies using isolated mitochondria, Ca^{2+} has been shown to activate both cholesterol side chain cleavage and 11β-hydroxylation (27, 33, 34) and also to increase the rate of P-450 reduction without an apparent change in the rate of reduction of adrenodoxin (34). Adrenocorticotropin activation of steroidogenesis is accompanied by acute increases in polyphosphoinositides which can avidly bind Ca^{2+} (35, 36). These phospholipids specifically stimulate mitochondrial cholesterol side chain cleavage (37) and may also affect Ca^{2+} levels in the immediate environment of the cytochromes $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$.

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