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Mitochondrial Cytochrome P-450$_{scc}$

MECHANISM OF ELECTRON TRANSPORT BY ADRENODOXIN*

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Complex formation between adrenodoxin and cytochrome P-450$_{scc}$ has been monitored via the increase in high spin cytochrome. A hyperbolic dependence upon adrenodoxin ($K_d = 8 \times 10^{-8}$ M) was changed to a sigmoid dependence by the addition of adrenodoxin reductase. The change is quantitatively explained by the change in the concentration of free adrenodoxin due to formation of a strong adrenodoxin reductase·adrenodoxin complex ($K_d = 1.8 \times 10^{-8}$ M) that does not significantly bind P-450$_{scc}$. No evidence was obtained for a ternary complex formation involving all three enzymes. Similarly, under steady state conditions, clear evidence was presented that a ternary complex is not a functional intermediate in electron transport. Cholesterol side chain cleavage dependence on adrenodoxin concentration showed an increasingly sigmoid pattern as the concentration of adrenodoxin reductase was increased. These plots could be transformed into Michaelis-Menten functions of free adrenodoxin ($K_m = 0.4$ μM) by assuming that adrenodoxin formed separate complexes with its reductase and cytochrome P-450$_{scc}$ and that a ternary complex did not form to any significant degree. Additionally, the present data could be most consistently interpreted, assuming at least a 5-fold decrease in the affinity of adrenodoxin reductase to adrenodoxin upon reduction of both enzymes. These results indicate that adrenodoxin transports electrons from adrenodoxin reductase to cytochrome P-450 by shuttling between these two enzymes.

During cholesterol side chain cleavage at saturating concentrations of adrenodoxin and adrenodoxin reductase, adrenodoxin was fully reduced and the reduction of adrenodoxin was not rate-limiting. At suboptimal concentrations of adrenodoxin reductase, a near steady state level of reduced adrenodoxin was rapidly established. The rate of cholesterol side chain cleavage did not correlate with the concentration of reduced adrenodoxin but instead correlated directly ($r = 0.965$) with the percentage of adrenodoxin reduced at the midpoint of the reaction. It is inferred that oxidized adrenodoxin inhibits cholesterol side chain cleavage by competition with reduced adrenodoxin for binding to P-450$_{scc}$ during the catalytic cycle.

The mammalian mitochondrial steroid and sterol monooxygenase systems characterized to date consist of three enzymes: a flavoprotein NADPH ferredoxin reductase, a ferredoxin, and a cytochrome P-450 (1-6). Adrenal cortex mitochondria contain a well characterized ferredoxin (adrenodoxin), a flavoprotein (adrenodoxin reductase), and two forms of cytochrome P-450; P-450$_{scc}$[1] and P-450$_{11\beta}$ which function in, respectively, cholesterol side chain cleavage and steroid 11β or 18-hydroxylation (1, 4-7). These mixed function oxidase systems resemble the camphor hydroxylase of *Pseudomonas putida* (8). There are close similarities between the sizes and amino acid contents of the mammalian and *P. putida* enzymes (4, 9). However, a major difference is that the mammalian cytochromes P-450 function as integral membrane proteins while P-450$_{cam}$ is a soluble protein (4, 5, 9).

Much of our knowledge of the catalytic cycle of monooxygenation has been derived from the extensive studies of camphor hydroxylation (8, 10). It is well established for camphor hydroxylation that reduced ferredoxin donates electrons to oxidized P-450$_{cam}$ and to a dioxygen complex of reduced P-450$_{cam}$, both complexed by substrate during a single catalytic cycle (8). Substrate binding raises the potential for the first electron transfer and accelerates this step while donation of the second electron is the slow step in the cycle (11, 12). The process of cholesterol side chain cleavage is further complicated by the need for three catalytic cycles for formation of pregnenolone (13). However, the initial mixed function oxidation step appears to be the rate-limiting step, at least in certain reconstituted side chain cleavage systems (14).

The electron transfer processes between NADPH and FLP and between FLP and ADX have been extensively studied (15, 16). Formation of high affinity complexes between FLP and ADX has been demonstrated (17). The recently developed purification procedures for two adrenal cortex mitochondrial cytochromes P-450 provide apparently homogeneous preparations (18-20). Hence, the interactions of ADX with a mitochondrial P-450 either in the absence or presence of FLP can now be studied under precisely defined conditions. Indeed, recent evidence has been presented that ADX can form a 1:1 complex with P-450$_{scc}$ (21) analogous to the complex formed between putidaredoxin and P-450$_{cam}$ (11). In addition, it has recently been shown that P-450$_{11\beta}$ also forms a complex with ADX which is weaker than the complex between ADX and FLP (22).

In this paper we examine the kinetics of cholesterol side chain cleavage activity in 0.3% Tween 20 which substantially stimulates the activity of the cytochrome (7). The dependence of SCC activity on the concentrations of ADX and FLP is compared with the formation of ADX·P-450$_{scc}$ complexes under the same conditions. Reaction kinetics are examined under two extreme conditions which facilitate simplification

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[1] The abbreviations used are: P-450$_{scc}$, cytochrome P-450 specific for cholesterol side chain cleavage; ADX, adrenodoxin; FLP, adrenodoxin reductase; SCC, cholesterol side chain cleavage; $K_{A/F}$, dissociation constant of the adrenodoxin reductase·adrenodoxin complex; $K_{A/P}$, dissociation constant of adrenodoxin·P-450 complex.

of the kinetics; high FLP concentrations with variable ADX or excess ADX with variable FLP. Evidence is provided that ADX does not bind FLP and P-450$_{scc}$ simultaneously to any significant degree and that formation of such a ternary complex is not obligatory for SCC activity. The results of our studies further indicate that in the presence of saturating FLP, all ADX molecules are reduced, and that the rate of SCC reaction is governed by a Michaelis-Menten function of the concentration of reduced ADX$_{free}$.

MATERIALS AND METHODS

Adrenodoxin was prepared according to the method of Orme-Johnson and Beinert except that Sephadex G-100 chromatography was substituted for the gel electrophoresis step (22). A final chromatography on Sephadex G-50 in 10 mM Tris buffer, pH 7.5, containing 0.5 M KCl improved the A_{280}/A_{415} ratio from 2.8 to 1.2. Adrenodoxin reductase was prepared according to the procedure of Hiwatashi *et al.* (23) to A_{272}/A_{450} = 8.4. Cytochrome P-450$_{scc}$ was purified to 13 nmol of P-450/mg of protein (A_{280}/A_{393} = 1.2) by a modification of the procedure of Suhara *et al.* (18) in which adrenodoxin-Sepharose affinity chromatography was added as a final step.

Cholesterol conversion to pregnenolone was assayed by a modification of the procedure of Takikawa *et al.* (24). All reactions were carried out in 25 mM of potassium phosphate, pH 7.2, containing 0.3% Tween 20 and 200 μM [^{3}H]cholesterol (SCC buffer) at 37°C for 4 min in a final volume of 0.2 ml. Incubations were terminated with 0.2 ml of ethanol. [^{14}C]Pregnenolone (1600 cpm) was added as a recovery standard and extraction was carried out with 2 ml of methylene chloride. The dry extract was dissolved in 40% methanol and [^{3}H]pregnenolone was separated from [^{3}H]cholesterol on minicolumns of Sephadex LH-20 (0.7 × 4 cm) with 50% methanol (33). For the spectrophotometric assays, the solutions were prepared exactly as for the cholesterol side chain cleavage assays except with a final volume of 1 ml. ADX in Fig. 1 and FLP in Fig. 3 were added in small volumes with a 10-μl Hamilton syringe. The spectrophotometric assays were carried out using an Aminco DW-2 spectrophotometer in dual wavelength (Fig. 1) or split beam (Fig. 3) modes.

Calculation of the free ADX concentration required solution of Equation 1 (see "Results") for ADX$_{free}$. This, however, is a cubic equation with only one real positive root. This root was determined with an interval halving algorithm in Fortran using a Sperry Univac 1110 computer. The statistical analyses of the data in Figs. 1 and 2 were carried out using the Fortran program HYPER (25) which fits the data to the hyperbola described by the Michaelis-Menten equation.

RESULTS

Adrenodoxin Binding to Flavoprotein and P-450$_{scc}$—When ADX binds to P-450$_{scc}$ in the presence of cholesterol, a major enhancement of the proportion of high spin P-450$_{scc}$ occurs. This change can be recorded as a type I spectral change and allows measurement of the binding of ADX to the low concentrations of P-450$_{scc}$ used in our standard side chain cleavage assay. In Fig. 1 (*inset*), the spectral response to increasing concentrations of ADX is shown together with the effect of two concentrations of FLP (1.56 and 3.12 × [P-450]). The addition of FLP causes a series of sigmoid curves which are displaced to the right in proportion to the concentration of FLP but which tend to the same ΔA_{max} at sufficiently high concentrations of ADX. The curve in absence of FLP when replotted as [ADX$_{free}$]$^{-1}$ *versus* [ADX-P-450]$^{-1}$ gives a straight line with a dissociation constant ($K_{A/F}$) equal to 8 × 10^{-8} M.

Previously, the complex between ADX and FLP has been shown to have a dissociation constant ($K_{A/F}$) of less than 10^{-8} M (17). Assuming that ADX, FLP, and P-450$_{scc}$ do not form a ternary complex, a possible explanation for the displacement of the complex-formation curves is that free ADX is decreased through complex formation with FLP. The additional ADX required to give a particular amount of P-450 complex in the presence of FLP then measures the amount of ADX bound to FLP at that concentration of ADX. In Fig. 1, these data are replotted as [FLP-ADX]$^{-1}$ *versus* [ADX$_{free}$]$^{-1}$ for both concentrations of flavoprotein. Statistical analysis indicated $K_{A/F}$ values of 1.8 ± 0.2 and 1.7 ± 0.4 × 10^{-8} M and maximum complex formation of 0.32 ± 0.01 μM and 0.66 ± 0.05 μM at, respectively, 0.25 and 0.50 μM added FLP. These data are then consistent with formation of a 1:1 FLP·ADX complex which does not bind significantly to P-450$_{scc}$.

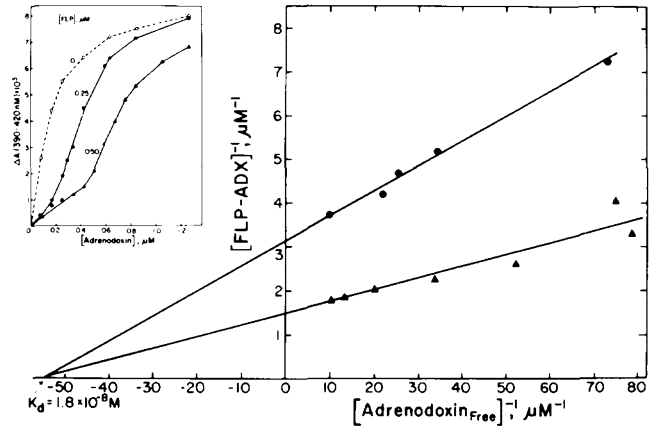

FIG. 1. **Adrenodoxin binding to P-450$_{scc}$ in the absence and presence of the flavoprotein as monitored by type I spectral change.** The reactions were carried out in 1 ml of SCC buffer containing 0.16 μM P-450$_{scc}$ without FLP (○- - -○), with 0.25 μM FLP (●——●) and with 0.5 μM FLP (▲——▲) at 35°C. The data are represented directly (*inset*) and as a plot of [FLP-ADX]$^{-1}$ as a function of [ADX$_{free}$]$^{-1}$.

Cholesterol Side Chain Cleavage and ADX Reduction—SCC activity was measured as a function of ADX concentration at four different FLP concentrations. As the concentration of FLP was increased, two superimposed effects were observed. Each activity plot was sigmoid and was shifted to the right with increasing concentration of FLP (Fig. 2*A*) as was seen with the formation of P-450·ADX complexes. In addition, the V_{max} activity increased somewhat with the concentration of FLP. This effect is seen more clearly in Fig. 3 where the dependence of SCC activity on FLP concentration is shown with a very large excess of ADX. SCC activity reaches a maximum of 17 to 18 nmol/nmol of P-450 min^{-1} at a ratio of FLP to P-450 of 0.5:1.

The visible absorption spectrum of the reaction mixture used in this experiment is dominated by adrenodoxin, particularly above 450 nm because of the large excess over P-450 (Fig. 3, *inset*). The time dependence of the spectral change during SCC was the same at all wavelengths between 450 and 660 nm so that changes in the spectrum of P-450 do not contribute significantly at this concentration of ADX. It is also clear that the level of reduced ADX changes in a biphasic manner. At a low concentration of FLP (13 nM), 30% of ADX is reduced within 1.5 min while the remainder reduces far more slowly ($t_{1/2}$ > 5 min). The spectrum from 450 to 650 nm at the completion of the NADPH-induced change was not significantly different from that of dithionite-reduced adrenodoxin. Except at the lowest concentration of FLP, the fast phase of reduction was complete within a short period relative to the SCC assay time (4 min). As the concentration of FLP increases, both the rate of rapid phase reduction and the proportion of ADX reduced during the rapid phase increase. In Fig. 3, we have plotted the proportion of ADX reduced at the midpoint of the SCC incubation (2 min) as a function of FLP concentration. There is clearly a high correlation (r = 0.965) between the percentage of ADX reduced and SCC activity.

Data in Fig. 2*A* have been replotted to determine the apparent K_m for ADX under conditions where excess FLP

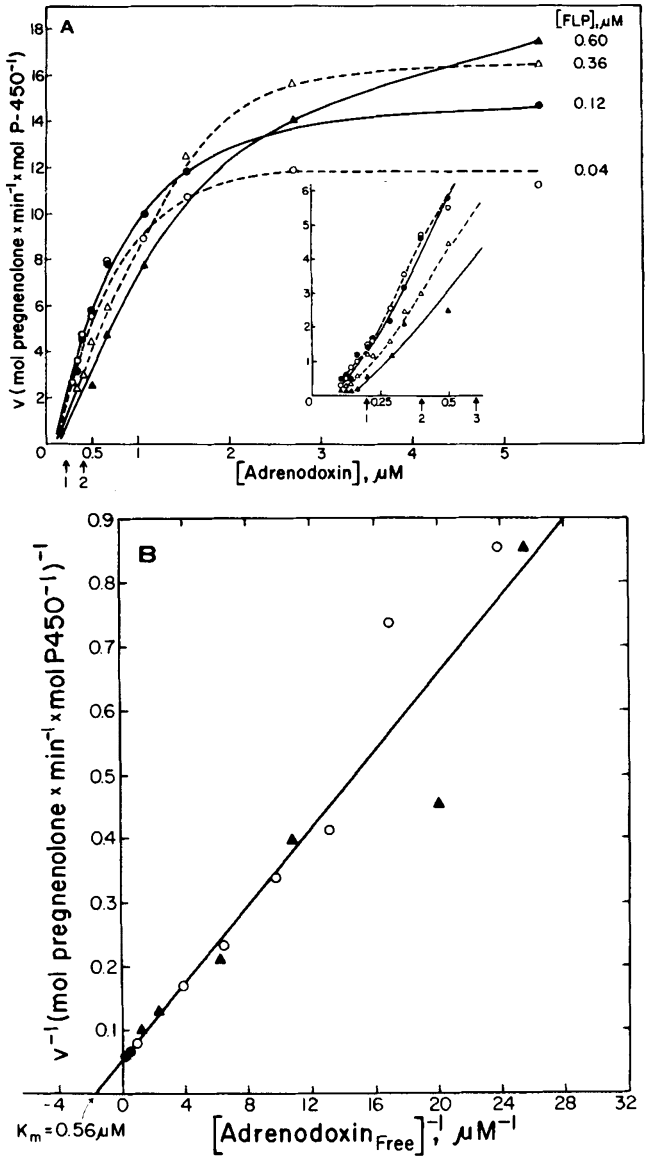

FIG. 2. **Cholesterol side chain cleavage dependence on adrenodoxin concentration.** *A*, cholesterol conversion to pregnenolone as a function of adrenodoxin concentration at four concentrations of FLP. The numbers below the *arrows* indicate [ADX]/[P-450$_{scc}$] ratios. The reactions were carried out in SCC buffer containing 0.2 μM P-450$_{scc}$ and 1 mM NADPH at 37°C. The *inset* presents the results from the same experiment at low [ADX]. *B*, Lineweaver-Burk plot of v^{-1} as a function of $[ADX_{free}]^{-1}$ in the presence of two saturating FLP concentrations, 0.36 μM (○) and 0.60 μM (▲). The values are based on data shown in *A*. ADX_{free} was calculated according to equation 1 under "Results." $K_{A/F}$ was taken as 9×10^{-8} M and $K_{A/P}$ as 8×10^{-8} M.

ensures complete reduction of ADX. Equation 1 has been used to calculate the concentration of free ADX.

$$ADX_t = ADX_f + \frac{FLP_t \times ADX_f}{ADX_f + K_{A/F}} + \frac{P\text{-}450_t \times ADX_f}{ADX_f + K_{A/P}} \quad (1)$$

The two complex terms represent the concentrations of, respectively, FLP·ADX and P-450·ADX complexes and subscripts *f* and *t* refer to, respectively, free and total enzyme concentrations. $K_{A/F}$ and $K_{A/P}$ represent the dissociation constants of the respective complexes.

When we used the values for $K_{A/F}$ and $K_{A/P}$ calculated from the equilibrium binding studies with oxidized enzymes (1.8×10^{-8} M, 8×10^{-8} M, respectively), a poor fit to the Lineweaver-Burk plot was obtained. However, strictly speaking, $K_{A/F}$ should correspond to the complex between reduced FLP and reduced ADX. Assuming that $K_{A/P}$ does not change, a good fit to a double reciprocal plot is obtained for both saturating concentrations of FLP when $K_{A/F}$ is increased 5-fold to 9×10^{-8} M (Fig. 2*B*). The fit again becomes poor when $K_{A/F}$ is increased more than 10-fold. However, during enzymatic turnover, $K_{A/P}$ must reflect the binding of reduced ADX to a mixture of P-450$_{scc}$ intermediates of the catalytic cycle. If $K_{A/P}$ decreases 6-fold upon reduction of ADX, as shown for the camphor hydroxylase system (11), then a good fit is obtained with a still higher $K_{A/P}$ value, *i.e.* 27×10^{-8} (data not shown as the line is very similar). The apparent K_m for ADX determined in this way was 0.56 ± 0.04 μM (Fig. 2B). It should be noted that this value is higher than the true K_m since cholesterol was not saturating. Other experiments involving apparent K_m determinations at varying levels of cholesterol indicate a K_m of about 0.4 μM.

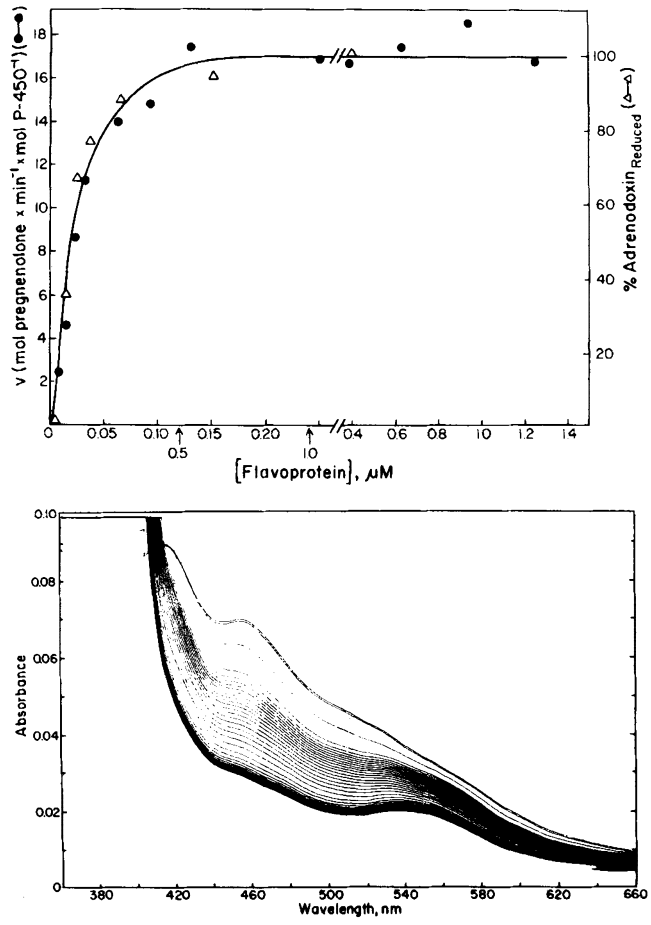

FIG. 3. **Cholesterol conversion to pregnenolone and relative amounts of reduced adrenodoxin as a function of flavoprotein.** *A*, The numbers below the *arrows* indicate [FLP]/[P-450$_{scc}$] ratios. Pregnenolone formation at 37°C (●) was initiated with 1 mM NADPH in SCC buffer containing 0.24 μM P-450$_{scc}$, 6.5 μM adrenodoxin, and selected concentrations of FLP. The percentage of ADX in the reduced state (△) was determined spectrophotometrically under the same conditions as were used for the activity assay. The decrease in absorbance at 454 nm, 2 min after the addition of NADPH (midpoint of enzymatic assay) is measured relative to the decrease caused by complete reduction by sodium dithionite. *B*, time dependence of adrenodoxin reduction with 13 nM FLP. The two spectra with highest absorption at 460 nm were recorded before and after the addition of FLP. After the addition of NADPH, scanning was started at a constant speed of 10 nm/s. The third spectrum reaches 460 nm at 20 s after mixing while subsequent spectra are at 1-min intervals.

DISCUSSION

Camphor hydroxylase of *P. putida*, steroid 11β-hydroxylation, and cholesterol side chain cleavage each utilize ferredoxins to mediate the transfer of reducing equivalents from a flavoprotein reductase to the cytochrome. This mechanism therefore fundamentally differs from microsomal P-450-catalyzed mixed function oxidation where direct electron transfer from the FLP occurs. The mode of operation of ferredoxins as a mediator has not been established. Oxidized ferredoxins form complexes with both P-450 and FLP; reduced putidaredoxin forms a complex with P-450$_{cam}$ prior to both first and second electron transfer steps (12). Two broad types of electron transfer mechanism may operate; electron transfer within a ternary flavoprotein·ferredoxin-P-450 complex or a shuttle mechanism in which the reduced ferredoxin transfers electrons between the flavoprotein and the cytochrome P-450. Lambeth *et al.* have provided evidence that 11β-hydroxylation prefers the second mechanism (21, 26). Here we have examined both possible mechanisms for cholesterol side chain cleavage by examining equilibrium complex formation and steady state enzymatic activity under the same conditions.

Ternary Complex Formation Is Undetectable—Excess FLP almost completely prevents the type I response of P-450$_{scc}$ to ADX. This alone does not exclude complex formation but indicates that at the least, FLP must drastically alter the interaction of ADX with P-450$_{scc}$. All but low levels of ternary complex can be ruled out by further quantitative evaluation of the spectrophotometric data. Thus, the maximal titration shift has proved to be equivalent within experimental error to the amount of added FLP. The value of $K_{A/F}$ (1.8×10^{-8} M) determined in this way is consistent with other determinations (26). Although we cannot completely rule out a ternary FLP·ADX·P-450$_{scc}$ complex, we calculate from these data that K_d for the complex with respect to FLP-ADX, must be at least 1 μM, *i.e.* 10 times weaker than for ADX-P-450$_{scc}$.

Data shown in Fig. 1 are analogous to that reported by Seybert *et al.* for cytochrome P-450$_{11\beta}$ (21). Their conditions differ in that they use a lower salt concentration which decreases $K_{A/F}$ (0.5×10^{-8} M) and higher concentrations of the proteins. If we assume that $K_{A/P}$ is the same for P-450$_{11\beta}$ and P-450$_{scc}$, then Equation 1 accounts for their data within experimental error. A similar sigmoid curve has been reported recently by Orme-Johnson and Light for the interaction of P-450$_{scc}$·cholesterol complex in the presence of FLP but absence of detergent.[2]

A Ternary Protein Complex Is Not a Significant Kinetic Intermediate—The saturation of side chain cleavage activity at low levels of reductase (Fig. 3) establishes that the rate is not dependent upon a weak ternary complex which might be undetectable in our binding studies. The effect of increasing [FLP] at low ADX also provides strong evidence against an intermediate ternary complex. Thus, when [ADX] is 1 to 3 times higher than [P-450$_{scc}$] an increase in [FLP] from 0.2 to 0.6 times [P-450$_{scc}$] actually decreases SCC activity, even though [FLP-ADX] increases approximately 3-fold. This can be explained by the competition of FLP with P-450$_{scc}$ for binding to ADX which implies that free ADX is a determinant of reactivity.

Activity Shows a Michaelis-Menten Dependence on Free Reduced ADX—Although ADX participates twice in each catalytic cycle, a double reciprocal plot of $1/v$ *versus* $1/[ADX_{free}]$ should be linear rather than parabolic because these two additions are not connected reversibly (27). For a shuttle mechanism in which reduced ADX transfers electrons from FLP to P-450$_{scc}$, activity should show a Michaelis dependence on the concentration of free reduced ADX. At high FLP concentrations where essentially all ADX is reduced in the steady state, the sigmoid activity curves in Fig. 2*A* can be converted into identical linear plots of $1/v$ *versus* $1/[ADX_{free}]$ (Fig. 2*B*). This fit requires at least a 5-fold increase in $K_{A/F}$ (9×10^{-8} M) which here refers to the interaction of reduced ADX with FLP (Fig. 2*B*). This weaker binding of reduced ADX to FLP is in agreement with the thermodynamic data of Lambeth *et al.* (16, 26).

Oxidized ADX Inhibits Activity—Oxidized ADX has a high affinity for P-450$_{scc}$ (Fig. 3*A*) (21) and therefore may be expected to compete with reduced ADX for the cytochrome. We indeed find that, with excess ADX, SCC activity correlates exactly with the steady state level of reduction of ADX. For example, in Fig. 3, a 20% decrease in reduced ADX and in SCC activity corresponds to an absolute change in $[ADX_{red}]$ from 6.5 μM to 5.2 μM. This concentration of reduced adrenodoxin would be fully sufficient to saturate P-450$_{scc}$ but for competition with the oxidized form. The relationship between activity and ADX_{red}/ADX_{total} at saturating levels of total ADX can be considered analogous to competitive product inhibition when substrate (ADX_{red}) + product (ADX_{ox}) is constant (28). In such a case with $ADX_{total} \gg K_m$, linearity is obtained if K_d for product, ADX_{ox}, is close to the K_m. On this basis, the binding of ADX_{ox} to P-450$_{scc}$ during enzymatic turnover is 10-fold higher than the K_d we have determined for the complex of oxidized ADX and P-450$_{scc}$.

The exact relationship between the kinetic constants so derived and binding equilibria for ADX is not presently clear. ADX_{ox} may compete with ADX_{red} at the sites of first or second electron donation. Since second electron transfer appears to be rate determining for P-450-dependent mixed function oxidations, it is likely that the kinetic constants are primarily determined by second electron transfer. Previously, it has been reported that oxidized putidaredoxin effects breakdown of the reduced dioxygen-adduct (29); thus, effective competition may occur at this point in the catalytic cycle.

When Is ADX Reduction Rate-limiting?—In mitochondria, the molar ratio of [FLP]:[ADX]:[P-450] appears to be approximately 1:10:10 (6, 30, 31). Thus, the reduction of ADX must be a rate-limiting step in mixed function oxidation, unless the turnover number for ADX reduction is at least 10 times higher than that for ADX oxidation by P-450. With low [FLP], steroid 11β-hydroxylation appears to be limited by ADX reduction (26). Under our conditions of optimal SCC activity, ADX reduction was not rate-limiting since the rate of reduction of ADX was at least 2 times the rate of oxidation of ADX_{red} attributable to SCC, and ADX reduction was rapidly completed. At present, however, it is not clear whether the similarity in the optimal [FLP]/[P-450] ratio (0.5 to 1) which has been found for SCC, camphor hydroxylase (32) and steroid 11β-hydroxylase (21) is coincidental or reflects a common mechanistic feature. This similarity is surprising in view of the very large range of monooxygenase activities encompassed by these enzymes.

If the present conditions reflect the situation in mitochondria, then the mitochondrial FLP concentration may provide a balance between the need for reduction of ADX molecules and the need to minimize competition between FLP and P-450 for binding to ADX. Competition between FLP and P-450 for binding to ADX would be further modulated by the decrease in the affinity of ADX to FLP (Fig. 2*B* and Ref. 26) and an increase in the affinity to P-450 upon its reduction (see "Results" and Ref. 11). The localized concentration of ions may also affect this process (26). The effect of the membrane

[2] N. R. Orme-Johnson and D. R. Light (1979) Abstract presented at the IV International Symposium on Microsomes and Drug Oxidations, Ann Arbor.

environment on these kinetic parameters remains to be determined.

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