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Electron Leakage from the Mitochondrial NADPH–Adrenodoxin Reductase–Adrenodoxin–P450scc (Cholesterol Side Chain Cleavage) System[1]

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In electron (e^-) transfer systems some e^- may "leak," reducing O_2 to a superoxide radical. This study examined the sites and kinetics of e^- leakage from the mitochondrial P450scc system. Adrenodoxin reductase alone oxidized NADPH, reducing O_2 to a superoxide radical at a very low rate. However, the reductase–adrenodoxin system reduced O_2 at a rapid steady-state rate with Michaelis–Menten dependence on [adrenodoxin](V_{max} = 3.5 μM e^-/min). After depletion of NADPH, reduced adrenodoxin was oxidized (autooxidation) with pseudo first order kinetics and the rate of e^- transfer decreased 10-fold. Ca^{2+} (<1 mM) stimulated e^- leakage in both phases. The reductase–adrenodoxin–P450scc system exhibited the highest rate of leakage (V_{max} = 7.8 μM e^-/min). At low [adrenodoxin] the majority of e^- leaked through P450scc and not through adrenodoxin. In the presence of the substrate, cholesterol, leakage drastically decreased to <0.5 μM e^-/min. These results indicate that the mitochondrial P450 systems can leak e^-, producing O_2 derived free radicals. Reduction of leakage during P450scc conversion of cholesterol to pregnenolone provides a clue to understanding physiological mechanisms that control e^- leakage. These may include coregulation of NADPH and cholesterol availability to the P450scc system and a system of antioxidants for quenching the oxygen radicals.

Mitochondrial P450 systems are located on the matrix side of the inner mitochondrial membrane and catalyze essential steps in the synthesis of steroid hormones, and many other steroidal compounds (1–5). The hydroxylation reactions catalyzed by these P450s require electrons which are transferred from NADPH to P450 by an electron transport chain that includes a flavoprotein (adrenodoxin reductase), and an iron–sulfur protein (adrenodoxin) in the following order:

NADPH → adrenodoxin reductase → adrenodoxin → cytochrome P450.

This electron transfer process may be "coupled" or "leaky." In a coupled system all electrons from NADPH are utilized in substrate hydroxylation reactions, whereas in a leaky system a portion of the electrons are transferred to other acceptors, such as O_2. Some of the microsomal cytochromes P450 are very leaky (6–8). The leakage of electrons outside of electron transfer chains appears to be a major source of oxygen radicals in cells (9). In some tissues the concentrations of the mitochondrial P450 system components are as much as 10 times higher than other electron transfer chain enzymes, such as microsomal P450s (10). Thus, even if these P450 systems leak electrons at a low rate, their total capacity for free radical generation could be high.

There are several indications that mitochondrial P450 systems may be a significant source of oxygen radicals: Electrons can be transferred directly from adrenodoxin reductase and adrenodoxin to O_2, resulting in the formation of superoxide radicals (11, 12). In steroidogenic cells in culture, some P450s can undergo rapid degradation in the absence of antioxidant vitamins (8, 13). The synthesis of steroid hormones is accompanied by a decrease in tissue levels of ascorbic acid (14, 15). A vitamin C deficient diet results in necrotic changes in the adrenal cortex (16). Among all organs in the body, the concentration of ascorbic acid is highest in the adrenal cortex (13, 17),

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a tissue which has the highest concentrations of mitochondrial P450s (2, 10). Thus, one possible role of the antioxidant vitamins in steroidogenic tissues may be the quenching of the radicals generated by the P450 systems.

The present study determines the sites and rates of electron leakage reactions in a reconstituted mitochondrial P450 system. Our results indicate that the mitochondrial P450 systems can leak electrons, producing O_2-derived free radicals. The formation of oxygen radicals is enhanced by P450scc, but inhibited in the presence of its substrate cholesterol. Diminution of electron leakage during P450scc hydroxylation of cholesterol provides a clue to understanding physiological mechanisms that control electron leakage in mitochondrial P450 systems.

MATERIALS AND METHODS

All reagents and enzymes were purchased from Sigma or Aldrich unless otherwise specified. [^{14}C]cholesterol (50 mCi/mmol) and [^{3}H]pregnenolone (10 Ci/mmol) were purchased from Amersham, England. Adrenodoxin reductase, adrenodoxin and P450scc were purified from bovine adrenal cortex (18–20). Concentrations of NADPH, adrenodoxin reductase, adrenodoxin, and P450scc were calculated from their extinction coefficients ($mM^{-1} \times cm^{-1}$) at 340 nm (6.2), 450 nm (10.9) 414 nm (11), and 390 nm (110), respectively. Absorbance was measured using a Kontron Uvicon 810 double-beam or a Hewlett–Packard HP-4851 diode-array spectrophotometer.

The spectrophotometric assays were carried out in a final volume of 0.5 ml in a thermostatically controlled cuvette. All reactions were started by the addition of adrenodoxin reductase. Unless otherwise indicated, all reactions were carried out at 37°C, in 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.2, under aerobic conditions. Bubbling the solutions with O_2 did not change the rate of electron leakage. Therefore, the solutions were used without this treatment which was employed in some previous studies. The NADPH oxidation rate was determined at 340 nm during the linear phase of the reaction. Since NADPH donates two electrons but adrenodoxin can be reduced only by a single electron, single electron transfer rate was expressed as NADPH oxidation rate multiplied by two. At 37°C, NADPH showed a slight degradation at a rate of 0.3 μM/min, and this value was subtracted in the calculation of the NADPH oxidation rate. The formation of superoxide radicals was detected by epinephrine oxidation at 480 nm (21) or reduction of nitroblue tetrazolium at 560 nm (22).

Oxygen radicals were detected using 5,5-dimethyl-1-pyrroline-1-oxide (DMPO)[3] as a spin-trap (23), which was purified on activated charcoal before use (24). The reactions were carried out in 100 μl in a flat sealed cell. Varian E-12 electron spin resonance (ESR) spectrometer was operated with the following conditions: magnetic field, 3400 G (full scale 100 G); frequency, 9.55 GHz; modulation amplitude, 0.8 G; microwave power, 20 mW; scan speed, 25 G/min; and time constant, 0.3 s.

Hydrogen peroxide was assayed using the Nash reagent (25, 26) with the following modifications: The reactions were carried out in 100 μl with 6% methanol, stopped by addition of 15 μl of 3 M trichloroacetic acid, and neutralized with 15 μl of 3.6 M KOH. Nash reagent (400 μl) was then added, and absorbance was measured after a 10 min incubation at 60°C. The Nash reagent was always freshly prepared and its reaction with 100 μl of reaction buffer containing 7% formaldehyde was checked to ascertain a constant reading from experiment to experiment. The concentration of H_2O_2 produced was estimated by comparing the experimental values to the standard curve using hydrogen peroxide. The amount of H_2O_2 used for the standard curve was determined by absorbance at 240 nm. Absorbance of the side reaction of dimethylformamide with the Nash reagent was subtracted.

Cholesterol side chain cleavage activity was assayed in a final volume of 100 μl as previously described (27) with some modifications: The reaction was stopped after 4 min by adding carrier [^{3}H]pregnenolone in 100 μl ethanol and 200 μl of petroleum–ether. The organic phase containing the steroids was removed and dried under nitrogen. The steroids were redissolved in methanol, and separated by isocratic high-pressure liquid chromatography on an Econosil C-18 reversed phase column (250 × 4.6 mm) with methanol:water, 95:5, at a flow rate of 0.8 ml/min. Cholesterol was detected at 219 nm and pregnenolone at 290 nm. Fractions were collected and counted in a Packard scintillation spectrometer.

Traces of transition metals were quantitated in samples of buffers and salt solutions using an inductively coupled plasma atomic emission spectrometer (Spectroanalytical Instrument, Kleve, Germany). The following results were obtained in all solutions examined: Cu < 0.15 ppm; Fe < 0.03 ppm; other metals < 0.02 ppm.

The K_m and V_{max} values were estimated by nonlinear regression analysis, fitting the data to the Michaelis–Menten equation using GraphPad Software (San Diego, CA) and the graphs were prepared using Stanford Graphics (3D-Visions, Torrance, CA).

RESULTS

Under the conditions of Fig. 1, the initial rapid phase of NADPH oxidation corresponded exactly to the time required for the complete reduction of adrenodoxin (Fig. 1B). The number of electrons donated by NADPH during this phase was very close to the number of adrenodoxin molecules reduced (adrenodoxin is a single electron acceptor).

During the second phase of NADPH oxidation (from 0.5 to 4 min in Fig. 1A) all adrenodoxin molecules remained reduced (Fig. 1B). This is referred to as the "steady-state phase of electron leakage," wherein adrenodoxin molecules are continuously being reduced and oxidized, transferring the electrons to O_2 (see below). During this phase adrenodoxin functions as an automatic "electron gun," constantly being loaded and fired, until electron supply (NADPH) is exhausted. As [NADPH] was increased, the duration of this steady-state phase also increased (Fig. 1C). With a catalytic concentration of reductase, a ratio of [NADPH]/[adrenodoxin] ≥4 was necessary to reduce 100% of adrenodoxin molecules during the steady-state.

After NADPH depletion, reduced adrenodoxin molecules oxidized at a slower rate (after 4 min in Fig. 1B and after 16 min in Fig. 1C) (Table I). This phase is referred to as the "autooxidation phase." At ratios of [NADPH]/[adrenodoxin] <4 all reduced adrenodoxin molecules reoxidized. When this ratio was increased, the proportion of molecules reoxidized (as monitored by absorbance at 414 nm) decreased as a result of free radical caused degradation of adrenodoxin (results not shown).

Oxygen as the Acceptor of the Leaking Electrons and Generation of Superoxide Radicals

In this system, depletion of O_2 in solution inhibited electron leakage and the reoxidation of adrenodoxin (Fig.

[3] Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; SOD, superoxide dismutase, adrenodoxin$_{red}$, reduced adrenodoxin; adrenodoxin$_{ox}$, oxidized adrenodoxin.

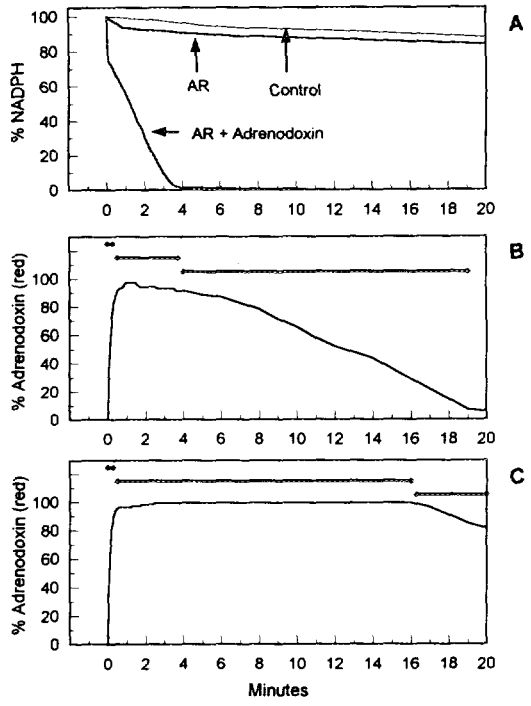

FIG. 1. NADPH oxidation by adrenodoxin reductase and adrenodoxin, and phases of electron leakage from adrenodoxin. Reaction conditions: 10 mM Hepes, pH 7.2, 50 mM KCl, 0.3 μM adrenodoxin reductase, and 11.8 μM adrenodoxin. [NADPH] was 48 μM in (A) and (B) and 83 μM in (C). The three horizontal bars in B and C mark the following phases: (i) Reduction of adrenodoxin, (ii) steady-state phase in the presence of NADPH, and (iii) autooxidation of reduced adrenodoxin after NADPH depletion.

TABLE I

The Rates of Electron Leakage to O_2 from Adrenodoxin Reductase, Adrenodoxin, and P450scc

Reactants	Rate
Steady-state	
A. reductase	<0.001 mol e^-/min/mol AR
A. reductase + adrenodoxin	149 ± 34 mol e^-/min/mol AR
A. reductase + adrenodoxin + P450scc	721 ± 125 mol e^-/min/mol AR
A. reductase + adrenodoxin + cytochrome c	387 ± 19 mol e^-/min/mol AR
Steady-state V_{max}	
A. reductase + adrenodoxin	3.5 ± 0.3 μM e^-/min
A. reductase + adrenodoxin + P450scc	7.8 ± 2.1 μM e^-/min
Auto-oxidation	
Adrenodoxin (reduced)	0.66 μM e^-/first min
Adrenodoxin (reduced)	0.13 (min) $^{-1}$

Note. All the rates are based on aerobic reactions carried out at 37°C, in 10 mM Hepes, pH 7.2, and 100 mM KCl. For comparison steady-state cytochrome c reduction was assayed with 0.05 μM adrenodoxin reductase, 1.8 μM adrenodoxin, 20 μM cytochrome c, and 50 μM NADPH (28). The turnover numbers (mean ± SD of three determinations) were determined under conditions where the rate of the reaction linearly increased with the concentration of reductase (AR) (see Figs. 5 and 10). The steady-state V_{max} are experimental (i.e., not extrapolated) values determined at saturating concentrations of the proteins indicated from two independent preparations. The rate of autooxidation for the first min is given for comparison with the V_{max} value; the second rate is the pseudo-first-order reaction constant (Fig. 6).

2). These results suggested that the electrons leaking from adrenodoxin, reduced O_2 converting it to superoxide anion (11). Reactions with epinephrine and nitroblue tetrazolium provided evidence for the formation of superoxide anions that could be inhibited with superoxide dismutase (SOD) (results not shown). However, these assays are not absolutely specific for superoxide detection (6).

To identify the superoxide anion more definitively we used the spin trap DMPO (23). In the presence of deferoxamine (a chelator that lowers the redox activity of transition metal ions) to prevent Fenton reaction, an ESR signal of the DMPO-OOH spin adduct was registered along with the DMPO-OH spin adduct (Fig. 3A). The DMPO-OOH signal disappeared in the presence of SOD (Fig. 3B).

As previously observed for the plant ferredoxin:ferredoxin reductase, and the microsomal P450 reductase (28, 29), Fe^{3+}–EDTA complex enhanced greatly the DMPO-OH signal produced by reductase alone or together with adrenodoxin (Fig. 3). The effect of reductase alone was observed at a high concentration of enzyme (1.7 μM in Fig. 3, vs 0.3 μM in Fig. 1). Adrenodoxin enhanced both the initial rate and the final level of the DMPO-OH signal (Fig. 3C). Catalase abolished the signal (Fig. 3C), indicating that the hydroxyl radicals were generated by the Fenton reaction (23). Addition of SOD in the absence of adrenodoxin, inhibited partially the reductase-produced signal. In contrast, SOD, in the presence of adrenodoxin, did not change the initial kinetic pattern (results not shown). This indicates that the catalytic Fe^{3+}–EDTA complex was reduced mainly by reduced adrenodoxin and not by superoxide anion.

The rate of electron flow increased as a function of [Fe^{3+}–EDTA]; as a consequence, the steady-state level of

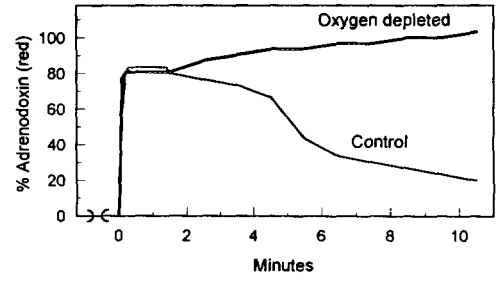

FIG. 2. Dependence of adrenodoxin autooxidation on oxygen. Reaction conditions: 10 mM Hepes, pH 7.2, 50 mM KCl, 0.3 μM adrenodoxin reductase, 12 μM adrenodoxin, and 24 μM NADPH. The reaction for oxygen depletion included 10 mM glucose and 35 U/ml glucose oxidase (the buffer solution was bubbled with nitrogen for 15 min before reductase addition).

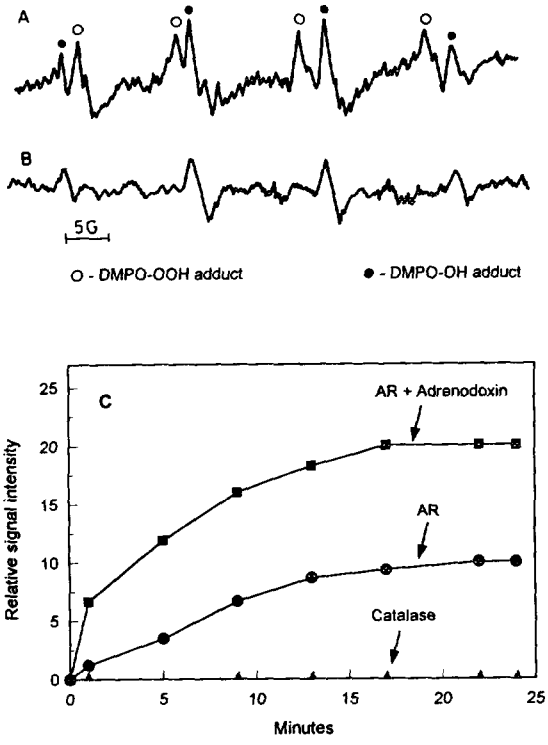

FIG. 3. (A and B) ESR spectra of DMPO oxygen radical spin adducts. Reaction conditions: 10 mM Hepes, pH 7.2, 100 mM KCl, 1.7 μM adrenodoxin reductase, 2.4 μM adrenodoxin, 1 mM NADPH, 100 μM deferoxamine, and 80 mM DMPO, at 25°C. (A) No additions; (B) with the addition of 40 μg/ml SOD. (C) Dependence of hydroxyl radical ($OH^{\cdot}$) formation on reductase and adrenodoxin in the presence of Fe^{+3}–EDTA complex. The $OH^{\cdot}$ was produced by the reaction of Fe^{+2}–EDTA complex with hydrogen peroxide. Reaction conditions: 10 mM Hepes, pH 7.2, 50 mM KCl, 1.7 μM adrenodoxin reductase, 1 mM NADPH, 10 μM $FeCl_3$, 20 μM EDTA, and 50 mM DMPO, at 25°C. (●) No additions; (■) with the addition of 2.4 μM adrenodoxin; (△) with the addition of 2.4 μM adrenodoxin and 10,000 U/ml catalase. The quantitation was based on the height of the second peak of the spectrum of DMPO-OH, which shows a quartet with the splitting constant a = 14.9 G and an intensity ratio of 1:2:2:1.

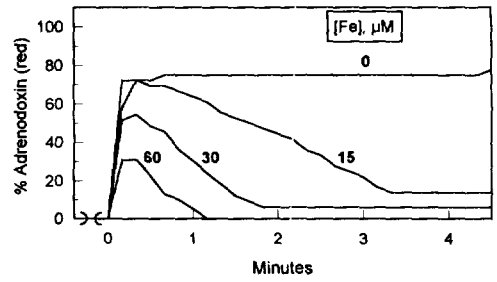

FIG. 4. Effect of Fe^{3+}–EDTA complex on electron leakage. Reaction conditions: 10 mM Hepes, pH 7.2, 100 mM KCl, 0.3 μM adrenodoxin reductase, 6 μM adrenodoxin, 30 μM NADPH, 200 μM EDTA, and $FeCl_3$ as indicated. Under these conditions, but in the absence of Fe^{3+}, autooxidation is seen only after 20 min and lasts longer than 15 min.

reduced adrenodoxin decreased, and both the steady-state and the autooxidation phases shortened (Fig. 4). Fe^{3+} without EDTA showed no effect at or below micromolar concentrations. Thus, the trace concentrations present in the buffer solutions (see Materials and Methods) were not responsible for any significant leakage.

The Kinetics of the Steady-State Phase of Electron Leakage

In the reductase–adrenodoxin system the maximal rate of NADPH oxidation was observed at a low concentration of reductase (20 nM), and increasing the [reductase] did not produce any increase in activity at near saturating [adrenodoxin] (>7 μM) (Figs. 5 and 6). The turnover number for the adrenodoxin dependent oxidation of NADPH was less than that for cytochrome c reduction (Table I). The cytochrome c reduction rate we observe is virtually identical to previously reported values (30, 31). Thus, the rate of electron leakage was not limited by the rate of electron transfer from NADPH to reductase. The saturation of activity at low [reductase] may be dictated by the rate of adrenodoxin reduction of O_2 vs rate of adrenodoxin interaction with reductase. With 20 nM reductase about 30% of the adrenodoxin molecules were reduced. Reduction of all adrenodoxin molecules were observed at >80 nM reductase. The fact that maximal electron leakage was observed in the presence of oxidized adrenodoxin (adrenodoxin$_{ox}$), indicates that adrenodoxin$_{ox}$ does not inhibit electron leakage. In contrast, adrenodoxin$_{ox}$ does inhibit P450 activity by competing with adrenodoxin$_{red}$ for binding to P450 (18, 20).

The dependence on [adrenodoxin] for O_2 reduction showed a good fit to Michaelis–Menten kinetics (Fig. 6), similar to reduction of nonphysiological quinones (32). At saturating concentrations of reductase (sufficient to maintain all adrenodoxin molecules reduced during the steady-state), the V_{max} did not vary, but increasing the

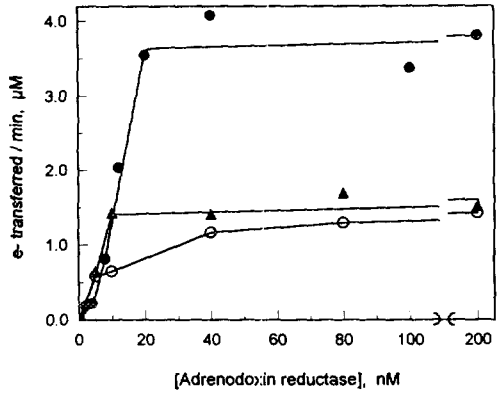

FIG. 5. Dependence of the steady-state phase of electron leakage on adrenodoxin reductase. Reaction conditions: 10 mM Hepes, pH 7.2, 100 mM KCl, and 65 μM NADPH. Adrenodoxin concentrations were (●) 7.2 μM, (△) 2.8 μM, and (○) 1 μM.

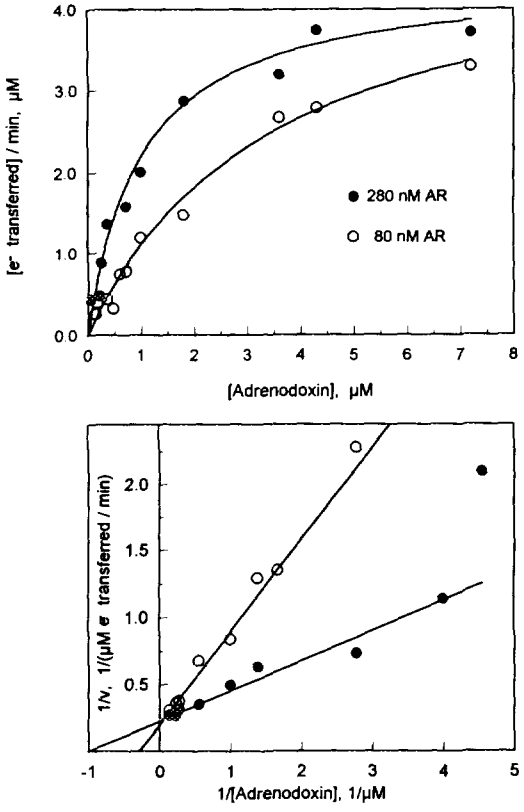

FIG. 6. Dependence of the steady-state phase of electron leakage on adrenodoxin. Reaction conditions: 10 mM Hepes, pH 7.2, 100 mM KCl, and 56 μM NADPH. Adrenodoxin reductase concentrations were (●) 280 nM and (○) 80 nM. (Top) hyperbolic curves are based on the Michaelis–Menten equation using K_m and V_{max} values estimated by a nonlinear regression analysis of the data. (Bottom) The lines are based on the same values of these two parameters: V_{max} = 4.4, and 5 μM/min; and K_m = 1 and 3.7 μM. Under these conditions the K_d for the reductase–adrenodoxin complex is about 0.5 μM (29).

[reductase] decreased the apparent K_m for adrenodoxin nearly fourfold (Fig. 6). The addition of 42 μg/ml SOD at saturating [adrenodoxin] did not affect the rate of the steady-state reaction.

The Kinetics of the Autooxidation Phase

The autooxidation of reduced adrenodoxin is a nonenzymatic process. We examined the fit of the data to first and second order reaction kinetics (33). Only a first order reaction (with respect to reduced adrenodoxin) showed a significant fit (Fig. 7). The rate of electron transfer from reduced adrenodoxin during the autooxidation phase was much slower than the turnover number during the steady state phase (Table I). The rate constant derived from this linear fit was highly dependent on temperature (data not shown). The activation energy calculated from the Arrhenius plot was 24.2 kcal/mol. This energy is higher than activation energies of most enzymatic reactions which are usually less than 20 kcal/mol (33).

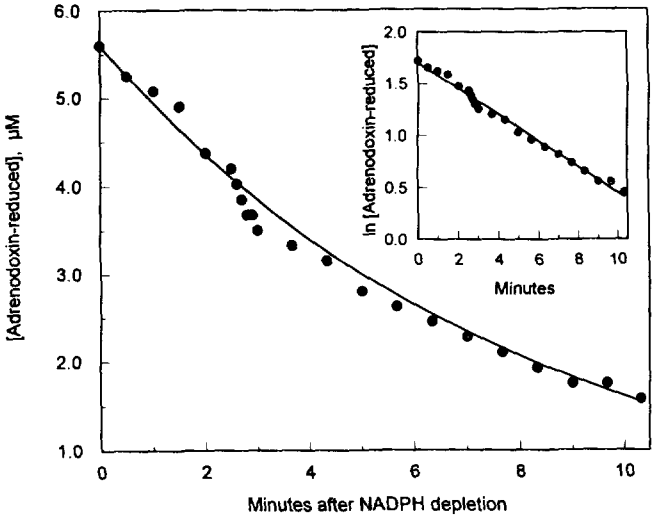

FIG. 7. The kinetics of the autooxidation phase. Reaction conditions: 10 mM Hepes pH 7.2, 100 mM KCl, 0.3 μM adrenodoxin reductase, 7 μM adrenodoxin, and 32 μM NADPH. [Adrenodoxin-reduced] was determined by absorbance at 414 nm. The points represent the actual data. The curve was fitted assuming a first order reaction with respect to adrenodoxin. (Inset) The log transformation of the data and the linear regression fit (r = 0.99).

Effect of Calcium Ions on Electron Leakage

Ca^{2+} ions stimulated electron leakage even at submillimolar concentrations (Fig. 8). Ca^{2+} increased the steady-state and the autooxidation rates, but in contrast to Fe^{3+}–EDTA complexes Ca^{2+} did not decrease the steady-state levels of reduced adrenodoxin (cf. Figs. 4 and 8). $MgCl_2$ had no effect on electron leakage at similar concentrations.

Electron Leakage in the Presence of P450scc

The addition of P450scc enhanced NADPH oxidation above the rate observed with reductase–adrenodoxin (Fig. 9). This effect reached saturation at 0.6 μM P450scc. P450scc is isolated as a complex with cholesterol that is tightly bound to the enzyme; yet, the metabolism of this

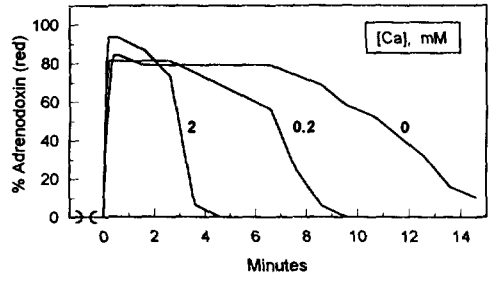

FIG. 8. Effect of Ca^{2+} ions on electron leakage. Reaction conditions: 10 mM Hepes, pH 7.2, 100 mM KCl, 0.3 μM adrenodoxin reductase, 2.8 μM adrenodoxin, 8.5 μM NADPH, and $CaCl_2$ as indicated.

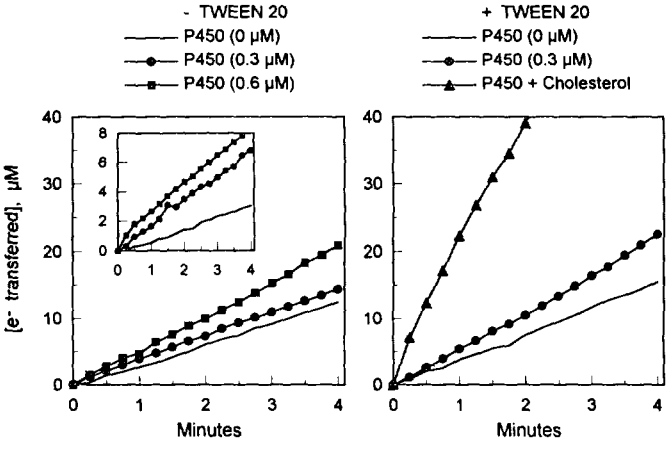

FIG. 9. Effects of P450scc and cholesterol on electron leakage in the absence (−) and presence (+) of the nonionic detergent Tween 20. Reaction conditions: 10 mM Hepes, pH 7.2, 100 mM KCl, 0.15 μM adrenodoxin reductase, 9 μM adrenodoxin (0.3 μM adrenodoxin for the experiments shown in the *inset* only), and 60 μM NADPH. P450scc, and cholesterol (200 μM) were added only in the experiments indicated. For the experiments with Tween 20 (0.3%) the reaction solutions were prepared as described (25). Under these conditions the rate of cholesterol side chain cleavage was determined as 14 mol pregnenolone/min/mol P450scc which equals to (14 × 6 × 0.15 nmol P450scc in 0.5 ml) 25.2 μM e^- transferred/min (cholesterol conversion to pregnenolone requires three monooxygenase reactions, i.e., six electrons; Ref. 35).

cholesterol would be responsible only for an insignificant portion of the total NADPH consumption. Thus, stimulation of NADPH oxidation reflected electron leakage and not catalytic activity. Control experiments showed that NADPH oxidation by reductase–adrenodoxin was not changed by the presence of 1% glycerol and 10 μM dithiotreitol in the reaction solution. Hence, the effect of P450 on NADPH oxidation was not caused by these chemicals added with P450scc which is kept in a buffer with 10% glycerol and dithiothreitol to stabilize the enzyme. P450scc showed the same magnitude of effect in K phosphate buffer as in Hepes buffer.

The stimulation of leakage by P450scc was not a simple additive effect over that of adrenodoxin. In the presence of P450scc, complete reduction of adrenodoxin was prevented even as [reductase] was increased (Fig. 10). This effect was analogous to the effect of Fe^{3+}-EDTA (Fig. 4). Apparently, P450scc decreased the steady-state level of adrenodoxin, as it rapidly oxidized adrenodoxin and prevented the reduction of all the molecules. This effect demonstrated that at low [$adrenodoxin_{red}$] electrons leaked mainly through P450scc. For example, in the absence of P450scc, the rate was about 1 μM e^-/min at [$adrenodoxin_{red}$] < 1 μM (Fig. 6); in the presence of P450scc leakage increased to above 4 μM e^-/min at the same [$adrenodoxin_{red}$] (10–13% adrenodoxin-reduced, Fig. 10). Reducing the concentration of total adrenodoxin reduced leakage, as would be expected by the Michaelis–Menten dependence. P450scc increased leakage even at low [adrenodoxin]. At equimolar concentrations of adrenodoxin and P450scc, as exist in adrenal mitochondria (10), the initial rate was stimulated threefold by the addition of P450scc (Fig. 9, inset).

Electron Leakage in the Presence of P450scc and Cholesterol

The effect of the P450scc substrate cholesterol could not be examined in the absence of phospholipids or detergents, as cholesterol solubility is very low in water (34). The solubilization of cholesterol by a nonionic detergent Tween 20 allows assay of the catalytic activity of P450scc (35). The activity of cholesterol side chain cleavage in 0.3% Tween 20 is even slightly higher than that in reconstituted phospholipid vesicles (20). To examine electron leakage under cholesterol turnover conditions we used this assay system.

In 0.3% Tween 20 containing buffer, the addition of P450scc increased NADPH oxidation over the level observed with reductase–adrenodoxin couple (Fig. 9). The inclusion of 200 μM cholesterol in Tween 20 produced no significant effect on NADPH oxidation by the reductase–adrenodoxin couple. However, in the presence of cholesterol, the reductase–adrenodoxin-P450scc system consumed the highest amount of NADPH. In this system, the rate of cholesterol conversion of pregnenolone was 14 mol pregnenolone/min/mol P450scc, requiring the transfer of 25.2 μM e^-/min. The observed rate of electron transfer was within 10% of this value, indicating that NADPH oxidation could be quantitatively accounted for, by the utilization of electrons for cholesterol metabolism, indicating that the rate of leakage was greatly reduced to <0.5 μM e^-/min (experimental error range).

Assay of H_2O_2 Production in the Presence of P450scc and Cholesterol

The results summarized above were based on the assay of the initial donor of electrons, NADPH, and the cho-

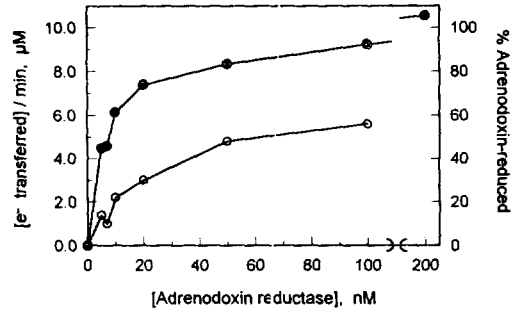

FIG. 10. Effect of adrenodoxin reductase on electron leakage (●) and percentage of adrenodoxin reduced (○) in the presence of P450scc. Reaction conditions: 10 mM Hepes, pH 7.2, 100 mM KCl, 8 μM adrenodoxin, 0.3 μM P450scc, and 60 μM NADPH.

lesterol side chain cleavage product pregnenolone. To assay the product of electron leakage, the superoxide anion, we carried out reactions under conditions wherein the superoxide radical would be completely converted to H_2O_2 by SOD. The H_2O_2 produced was then assayed using the Nash reagent.

This assay showed that addition of P450scc without substrate stimulated the rate of electron leakage observed with the reductase–adrenodoxin couple (Fig. 11). The addition of the substrate, cholesterol, drastically reduced electron leakage. The presence of SOD and other components of the reaction mixture increased the rate of leakage compared to standard conditions, but did not significantly affect cholesterol side chain cleavage activity.

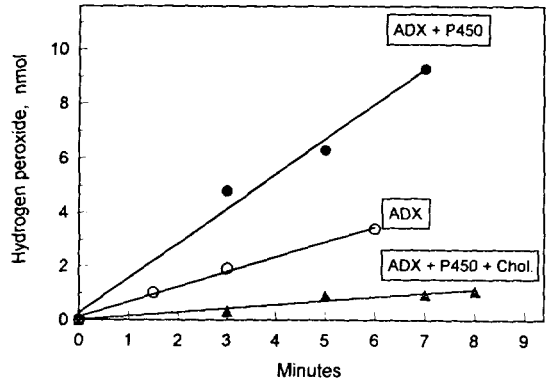

FIG. 11. Effects of P450scc and cholesterol on H_2O_2 production. Reaction conditions: 10 mM Hepes, pH 7.2, 100 mM KCl, 0.3% Tween 20, 1 mM NADPH, 0.25 μM adrenodoxin reductase, 9 μM adrenodoxin, 60 μM deferoxamine, 42 μg/ml SOD, 500 U/ml catalase, and 6% methanol. P450scc (0.3 μM) and cholesterol (200 μM) were added only in the experiments indicated. All reactions also included 1% glycerol, 5 μM dithiothreitol (present in P450 solutions), and 2% dimethyl formamide (solvent for cholesterol), to equalize the conditions for all reactions.

DISCUSSION

This study shows that the mitochondrial P450 system can oxidize NADPH in the absence of substrate, transferring electrons to O_2 and producing oxygen radicals. Major sites of leakage in the electron transfer chain are identified, and strong inhibitory influence of substrate metabolism on this leakage is demonstrated. The roles of these and other factors and their biochemical significance are discussed below.

The Roles of Adrenodoxin Reductase and Adrenodoxin in Electron Leakage in the Absence of P450

Adrenodoxin reductase does not represent a major source of electron leakage in the absence of adrenodoxin (Table I). Adrenodoxin reductase by itself can reduce O_2 only at a relatively low rate which can be detected at a high concentration of enzyme (Fig. 3; Refs. 11, 36). Adrenodoxin greatly stimulates the rate of NADPH oxidation and O_2 reduction (Figs. 2 and 3). However, the rates we observe (Table I) are more than an order of magnitude lower than those of a previous report (12).

During the steady-state phase, adrenodoxin_{red} may reduce O_2 in unbound form or while bound to reductase. The results indicate that binding of adrenodoxin to reductase does not block its reduction of O_2. In contrast, in similar experiments reductase inhibits P450 catalytic activity by competing with P450 for binding to adrenodoxin (18, 20, 37) as the sites of binding for these two proteins apparently overlap (38, 39). The possibility that O_2 is reduced by the reductase_{red}–adrenodoxin_{red} complex is also consistent with the redox potentials. The redox potential of adrenodoxin in unbound form (−290 mV) is shifted to −360 mV upon its binding to reductase (the redox potentials vary with pH and ionic strength; their significance for the present discussion lies in the shift to a more negative value by >−70 mV) (31). The redox potential for the O_2/O_2^- couple is −330 mV (40, 41). Thus, the complex would have a more favorable potential to reduce O_2 than unbound adrenodoxin_{red}.

The kinetic analyses show that at low adrenodoxin concentration the rate limiting step is dependent on the level of adrenodoxin reductase. Thus, the V_{max}/K_m ratio for adrenodoxin (the apparent first order rate constant at low [adrenodoxin]) is proportional to reductase concentration, and adrenodoxin reduction is the slow step at low [adrenodoxin]. The fact that the V_{max} is not dependent on [reductase] while V_{max}/K_m ratio is, results in the apparent K_m being dependent on [reductase]. However, this is probably the result of some other step becoming rate limiting at high [adrenodoxin] rather than a true change in affinity.

Although, the initial velocity of electron leakage is strongly dependent on adrenodoxin reductase and adrenodoxin, the V_{max} is independent of the levels of these proteins (see Figs. 5 and 6). The concentration of O_2 (~240 μM) cannot be limiting the V_{max} of 4 μM e^-/min. The rate of electron transfer to O_2 is lower than the rate of cytochrome c reduction by reductase and adrenodoxin (Table I). Thus, the rate limiting step in electron flow to O_2 is most likely the reduction of O_2 and not any of the prior steps of electron transfer. Currently we do not know what limits the V_{max} for this reaction.

The Kinetics of Autooxidation of Reduced Adrenodoxin

Immediately after NADPH depletion, the rate of electron leakage from adrenodoxin_{red} (autooxidation) drops nearly 10-fold (Table I). To explain this sudden decrease three changes may be considered to take place at the transition from the steady-state to autooxidation phase:

1. Change in the pathway of electrons: During the steady-state phase electrons proceed through reductase, adrenodoxin and finally to O_2. The kinetic analyses in-

dicate that this process can occur within a reductase–adrenodoxin complex, whereas during autooxidation electrons have to be transferred directly from adrenodoxin. If in both steady-state and autooxidation phases unbound adrenodoxin$_{red}$ was reducing O_2 directly then similar rates would be expected for both phases. The different rates observed indicate that the pathway of electron transfer and/or molecular interactions of adrenodoxin with O_2 are also different in the two phases.

2. Oxidation of reduced reductase: During the steady-state phase, reductase is completely reduced. Upon depletion of NADPH, reduced reductase would be rapidly oxidized by adrenodoxin leaving all reductase molecules in the oxidized state. If the affinity of adrenodoxin were different for the reduced versus oxidized forms of reductase then the change in the redox state of reductase may provide an explanation.

3. Change in NADPH concentration: If the rate of electron leakage depended on NADPH concentration, then NADPH oxidation rate should slow as [NADPH] decreased. However, the rate of NADPH oxidation was observed to proceed linearly until NADPH was completely oxidized (under conditions wherein the free radical mediated damage to adrenodoxin was insignificant, Fig. 1). Thus, NADPH depletion alone cannot be responsible for the decrease in the rate.

The Effect of Metal Ions on Electron Leakage from the Reductase–Adrenodoxin Couple

Electron leakage from the reductase–adrenodoxin couple was highly stimulated by Fe^{3+} and Ca^{2+} ions by different mechanisms. The soluble Fe^{3+}–EDTA complex (but not Fe^{3+} by itself) greatly stimulated e^- leakage as a result of rapid reduction of Fe^{3+} directly by adrenodoxin$_{red}$. Since Ca^{2+} cannot be reduced by a single e^-, its stimulatory effect reflected a direct enhancement of the rate of e^- transfer probably at the stage of adrenodoxin$_{red}$ reduction of O_2. Ca^{2+} has been implicated in membrane lipid peroxidation, but its effects are generally considered to be indirect via other intracellular processes (42). This study demonstrates that Ca^{2+} leads to increased oxygen radical formation by directly enhancing e^- leakage from an electron transfer chain.

The fact that Ca^{2+} (<1 mM) stimulates e^- leakage provides one explanation for Ca^{2+} (also <1 mM) inhibition of P450scc activity reconstituted in Tween 20 or in phospholipid vesicles (20): The stimulation of e^- flow to O_2 reduction would inhibit the flow of electrons to P450 for use in cholesterol hydroxylation. A previous study reported that Ca^{2+} stimulates the activity of P450scc in the absence of KCl (43). The effect of Ca^{2+} on P450scc activity depends on ionic strength (20). Hence, the effects of Ca^{2+} at physiological range of ionic strength (at 100 mM KCl or NaCl as in this study and in Ref. 20) may be physiologically more relevant.

Ca^{2+} is essential for the hormonal stimulation of steroidogenesis, and trophic hormone action in cells is accompanied by changes in Ca^{2+} fluxes from mitochondria (44, 45). The effects of Ca^{2+} on the reconstituted enzymes may reflect *in situ* regulatory effects of Ca^{2+}. However, extrapolation from studies with purified enzymes to *in situ* events is difficult at present.

The Role of P450scc in Electron Leakage

P450scc was more active than adrenodoxin in promoting electron leakage to O_2, and its maximal effect was observed at lower concentrations (0.3 μM vs > 7 μM for adrenodoxin). In adrenal mitochondria the concentrations of P450scc and adrenodoxin are similar (10). In a system with this molar ratio of the two proteins, electrons appeared to leak mainly through P450scc and not through adrenodoxin, as schematically shown in Fig. 12. Since

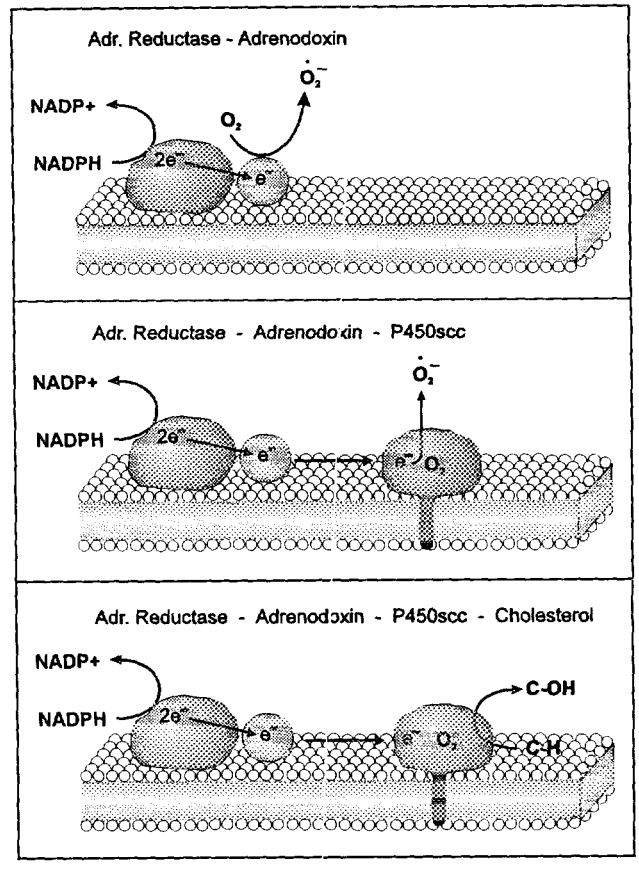

FIG. 12. Major pathways of electron (e^-) transfer from NADPH to the final acceptor in reconstituted mitochondrial P450scc systems. (Top) With adrenodoxin reductase and adrenodoxin system, electrons predominantly leak through adrenodoxin. The major acceptor of electrons is oxygen which gets converted to superoxide radical. (Middle) With adrenodoxin reductase, adrenodoxin and P450scc system, electrons mainly leak through P450scc. (Bottom) In the presence of the substrate cholesterol, leakage is very low, and electrons are mainly utilized in substrate hydroxylation (see text).

under anaerobic conditions the reduction potential of substrate free P450scc (−412 mV) is lower than that of adrenodoxin (−290 mV) (46), it was suggested that substrate free P450scc should not be readily reducible by reduced adrenodoxin (37, 47). However, this study establishes that substrate free P450scc can be reduced easily under aerobic conditions. A possible explanation for this apparent discrepancy is that the binding of O_2 and adrenodoxin increases the redox potential of P450scc. Many microsomal P450s have been observed to function as NADPH oxidases reducing oxygen in the absence of a substrate and producing superoxide anion, hydrogen peroxide, or water (6–8). NADPH oxidase activity of some of these microsomal P450s (e.g., Ref. 7), is much higher than that of P450scc.

The Role of P450scc Substrate Cholesterol in Inhibition of Electron Leakage

In a fully reconstituted P450scc system catalyzing cholesterol conversion to pregnenolone, electron leakage was greatly inhibited (Figs. 9 and 11). This coupling of electron transfer may be explained by the following observations: (a) cholesterol enhances the binding of adrenodoxin to P450scc (cholesterol and adrenodoxin display mutual positive cooperativity, and both shift the heme Fe(III) into the high spin state) (19, 47), (b) cholesterol binding increases the redox potential of P450scc (46, 47), and (c) the binding of cholesterol and its hydroxylated derivatives stabilize the oxyferro complex of P450scc (48). On theoretical considerations it has been noted that the first two mechanisms may operate "to prevent the hemoprotein from functioning as an NADPH–oxidase/oxygen reducing system" (37). One possible explanation for the substrate-dependent inhibition of leakage from P450scc is that, as observed in the P450cam structure (49), the substrate may fully occupy the active site, excluding water and decreasing the polarity of the microenvironment of the FeO_2 complex, and consequently inhibiting the release of O_2^-. In other P450 systems, various substrates and substrate analogs show wide variation in their effects on the coupling of the electron transfer process (6–8).

Is NADPH Availability to the Mitochondrial P450 System Regulated?

The present findings raise several questions regarding the function of the P450 system within the mitochondria: Is NADPH constantly available to the P450 systems even in absence of the substrate cholesterol? If so, is adrenodoxin constantly kept in a reduced state, and does it or P450 leak electrons? If the proteins do leak electrons then what types of cellular mechanisms operate to control the damaging effects of the oxygen radicals?

If the leakage observed with purified proteins reflect their functioning within the mitochondria, then based on the present findings the hypothesis could be raised that, in order to minimize electron leakage, NADPH synthesis or availability may be coregulated with cholesterol availability. The delivery of cholesterol to P450scc is regulated by trophic hormones in steroidogenic tissues and represents the rate-limiting step in acute stimulation of steroidogenesis (4, 5, 50). Several early studies provided evidence that ACTH also regulates availability of NAPDH (51). In contrast, observations that adrenodoxin is fully reduced in hypophysectomized rat adrenals with or without ACTH treatment, were interpreted to minimize the role of ACTH regulation of reducing equivalent supply (52). This interpretation may not be warranted, as cellular metabolism could have changed within minutes after induction of anesthesia and prior to the removal of the adrenal. Soluble cholesterol analogs, such as 20α- or 25-hydroxy-cholesterol, which can penetrate mitochondrial membranes and support adrenocortical steroidogenesis without the rate limiting step of cholesterol delivery (3, 50, 53, 54) may be useful tools for analyzing regulation of NADPH generation independent of cholesterol supply.

NADPH that supplies electrons to the mitochondrial P450 systems may be generated by several alternative routes (5, 50, 54–56). One main route in the bovine adrenal is controlled by the malic enzyme located in the matrix, and addition of malate to isolated mitochondria increases the levels of reduced adrenodoxin even in the absence of P450 substrate (55). Thus, supply of NADPH to the mitochondrial P450 systems may have to be regulated at the level of reducing precursors. Further study of the regulation of NADPH synthesis and availability is necessary to determine the biochemical significance of the reactions reported in this study.

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