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Enzymatic Mechanism of Steroid Hydroxylation

Reduced nicotinamide-adenine dinucleotide phosphate serves two distinct roles in hydroxylation.

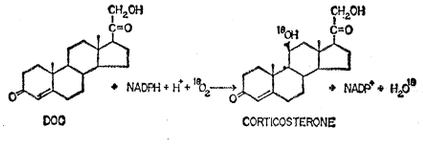
Charles J. Sih

Enzymes that incorporate one atom of molecular oxygen into substrates while concomitantly reducing the other atom to water are termed mixed-function oxidases (*1*) or monooxygenases (*2*). The stoichiometry of this type reaction may be represented as follows:

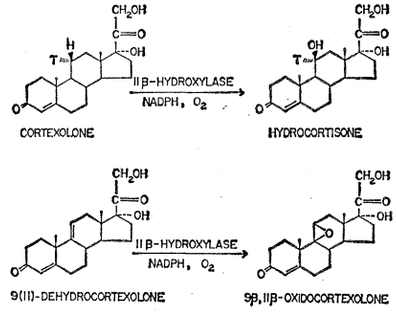
$$RH + O_2 + H_2A \longrightarrow R\text{–}OH + A + H_2O$$

RH is the substrate, and in most instances, the reductant; H_2A is either reduced nicotinamide-adenine dinucleotide phosphate (NADPH) or reduced nicotinamide-adenine dinucleotide (NADH). The importance of monooxygenases in metabolism is evident from the wide variety of substances they attack—such as, carbohydrates, lipids, amino acids, drugs, and hormones. Although considerable efforts have been devoted in the past decade to defining the enzyme components, coenzymes, and cofactors required for oxygenation, the mechanism of action of monooxygenases on a molecular level remains poorly understood. Studies on the properties of monooxygenases have been complicated by the particulate nature of the enzyme components and their relative instability. However, some progress has been made in recent years with the steroid 11β-hydroxylase (E.C.1.14.1.6) system of adrenal cortex mitochondria. In this article, the mode of action of monooxygenases with special reference to the functional roles of the electron donor NADPH is discussed.

The hydroxylation of deoxycorticosterone (DOC) at carbon-11β by adrenocortical mitochondria requires NADPH (*3*) and has been studied by the incorporation of ^{18}O from molecular oxygen into the substrate molecule, DOC (*4*). Although the stoichiometry for NADPH in this reaction is not established, there is nearly a one-to-one correlation between oxygen consumed and DOC hydroxylated (*5*). Therefore, the 11β-hydroxylase of adrenal cortex mitochondria resembles a classical mixed-function oxidase.



Enzymatic hydroxylation of steroids is known to proceed with retention of configuration (*6*), a course reminiscent of electrophilic displacement reactions. Steroid hydroxylases appear to obey the rule of Bloom and Shull (*7*) that a hydroxylase which is capable of introducing an axial hydroxyl at a given carbon atom (C-n) is also capable of converting a dehydro-substrate into an oxide also "axial" at C-n. For example, incubation of 9(11)-dehydrocortexolone with the 11β-hydroxylase of adrenal cortex mitochondria in the presence of NADPH and O_2 results in the formation of 9β,11β-oxidocortexolone (*8*). Furthermore, it has been shown that the dehydrosteroid and the corresponding saturated steroid compete for the same enzyme site (*9*). On the basis of these observations, it is likely that the so-called "active oxygen" in enzymatic hydroxylations may be the electrophilic OH^+ species.



Although the requirement of several components for steroid hydroxylation had been reported (*10*), the functional role of these components remained unknown until the studies of Omura and co-workers (*11–13*) and of Kimura and his group (*14–16*). The mitochondrial 11β-hydroxylase system has now been resolved into three components: flavoprotein or adrenodoxin reductase; nonheme iron protein or adrenodoxin; and hemoprotein or cytochrome P-450 (P-450).

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Adrenodoxin-reductase

The enzymatic reduction of adrenodoxin by NADPH is catalyzed by the enzyme, adrenodoxin-reductase. This enzyme is associated with mitochondrial particles and can be partially released by sonication (*17*). The extremely low concentration of this enzyme in the adrenal cortex mitochondria has hampered its purification. From 1 kilogram of beef adrenal cortex, one can obtain about 7 to 8 milligrams of this flavoprotein with a specific activity of about 500 (nanomoles of 2,6-dichlorophenol indophenol reduced per minute per milligram of protein) (*13*). The absorption spectrum of the most purified preparation which is typical of a flavoprotein is characterized by the decrease in absorbance at 450 and 460 nm upon reduction by dithionite. Adrenodoxin-reductase has a molecular weight of about 60,000, as determined by gel filtration, and contains 1 mole of flavin adenine dinucleotide as the prosthetic group with no apparent metal component (*16*). Although either NADPH or NADH could serve as the electron donor, the rate of reduction of adrenodoxin with NADPH was about 400 times faster than with NADH. Adrenodoxin-reductase cannot be replaced by photosynthetic pyridine nucleotide reductase (*18*) or NADPH-cytochrome c reductase from pig liver (*19*), an indication that the the NADPH-adrenodoxin reductase system is quite specific.

Adrenodoxin

This nonheme iron protein was isolated from adrenal cortex mitochondria of beef and pig (*12, 16*) and recently from pig testis (*20*). Like all nonheme iron proteins, adrenodoxin binds tightly to diethylaminoethylcellulose, facilitating its isolation. The yield of adrenodoxin is about 7 to 15 milligrams from 1 kilogram of beef adrenal cortex (*17*). The purified brown adrenodoxin is homogeneous upon ultracentrifugation, with a sedimentation constant ($S_{20,w}$) of 1.55*S*; it has a molecular weight of about 13,000 to 15,000 (*21*) and contains two atoms of iron and 2 moles of labile sulfide per mole of protein. The optical spectrum of oxidized adrenodoxin displays three absorption maximums at 455 nm, 414 nm, and 320 nm, with a small maximum at about 280 nm. After enzymatic reduction by NADPH and adrenodoxin reductase, the absorption in the visible spectrum is bleached about 50 percent; the original oxidized spectrum can be slowly restored by the introduction of air, indicating that the reduced form of adrenodoxin is autooxidizable. Electron resonance spectroscopy reveals that reduced adrenodoxin has a prominent, axially symmetric signal with $g_{\perp} = 1.94$ and $g_{\parallel} = 2.01$ (*22*). When the iron and sulfur in other nonheme iron proteins are substituted with isotopes Fe^{57} and S^{33}, both sulfur and iron contribute to the signal at $g = 1.94$ (*23*). Quantitative evaluation of the electron resonance spectroscopy signal by double integration indicates that about 50 percent of the iron in adrenodoxin is enzymatically reducible. However, titrations of adrenodoxin with NADPH and adrenodoxin reductase potentiometrically reveal that adrenodoxin is a two-electron acceptor and suggests that one iron atom and another oxidant in the protein are reduced. Titration of adrenodoxin with *p*-chloromercuribenzoate reveals the presence of four mercurial reactive groups per iron atom and suggests the possible involvement of two cysteinyl sulfhydryl groups and one labile sulfide group. Results from proton relaxation measurements (*24*) suggest that the iron atoms reside in the matrix of the protein, and are coordinated by nitrogen and sulfur ligands; water molecules are absent in the coordination sphere of the iron.

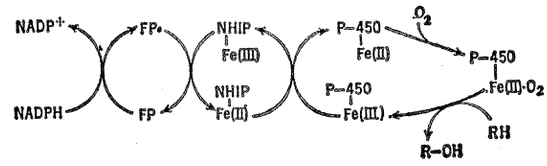


Fig. 1. Schematic representation of the hypothetical role of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) in hydroxylation reactions. Abbreviations are: FP, adrenodoxin reductase; NHIP, adrenodoxin; P-450, cytochrome P-450; RH, substrate.

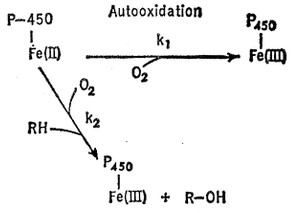
Cytochrome P-450 (P-450)

This type of carbon monoxide-binding hemoprotein was first found in liver microsomes (*25*) and subsequently in adrenal microsomes (*26*) as the terminal oxidase in mixed-function oxidative reactions, such as drug metabolism and steroid C-21 hydroxylation. Harding's group (*27*) first reported the presence of a pigment of the cytochrome P-450 type in adrenal cortex mitochondria and suggested that it may serve as the terminal oxidase for the 11β-hydroxylation of DOC. With the use of the photochemical action spectrum technique of Warburg (*28*), the rate of conversion of DOC into corticosterone in the presence of light was compared to the rates obtained in the darkness in the presence and absence of CO. It was observed that light reversal of the CO-inhibition was maximum at 450 nm (*29*). Furthermore, half inhibition by CO of DOC hydroxylation occurred when the ratio of CO to O_2 was 1. These findings led to the assignment of cytochrome P-450 to the CO-binding pigment of adrenal cortex mitochondria as the oxygen-activating enzyme in 11β-hydroxylation of steroids. Cytochrome P-450 has resisted purification because of its particulate nature and its facile conversion into an inactive form, cytochrome P-420 (P-420), with an absorption maximum at 420 nm under an atmosphere of CO. A variety of agents catalyze the conversion of P-450 into P-420. These include: ureas, amides, ketones, nitriles, monohydric alcohols, quanidium salts, cholate, sodium dodecyl sulfate, and phospholipase (*30*). However, P-420 can be converted back to P-450 by treatment with polyols such as glycerol or reduced glutathione (*31*). Also, P-450 can now be stabilized for more than 1 week in the presence of 20 percent (by volume) glycerol. In general, two types of change in the difference spectrum of P-450 are produced by various substrates: Type I is characterized by a trough at about 420 nm and a peak at about 388 nm; type II is characterized by a peak at about 430 nm and a trough at about 394 nm (*32*). After the addition of DOC to adrenal cortex mitochondrial preparations, the change in the type I spectrum was observed in the difference spectrum (*33*). It is still unclear whether these spectral shifts are due to an alteration in ligand binding to the heme of P-450 or to allosteric transitions of the hemoprotein (*34*). Evidence seems to favor the idea that P-450 is largely in the oxi-

dized form during the aerobic steady state of hydroxylation but no conclusive evidence is available as to whether DOC combines with the oxidized or reduced form of P-450. The nature of the prosthetic group of P-450 from adrenal cortex mitochondria is unknown, but protoporphyrin IX has been identified as the prosthetic group of P-450 from *Pseudomonas* (*35*).

On the basis of reconstitution experiments, the following roles in electron transfer and steroid hydroxylation have been proposed for these three components of adrenal cortex mitochondria (*12, 16, 36*) (Fig. 1). Electrons from NADPH are transferred to P-450 through adrenodoxin reductase and adrenodoxin. The reduced P-450 thus generated reacts with DOC and molecular oxygen to yield an activated complex, RH-P-450-Fe(II)-O_2, which is then converted into the product R-OH and H_2O. During the hydroxylation process, P-450-Fe(II) is oxidized to P-450-Fe(III); the function of NADPH in this model is solely to provide the reducing equivalents for P-450-Fe(III). This reaction scheme appears to be the currently accepted view for several mixed-function oxidases (*37–39*).

The stoichiometry of oxygen consumption, NADPH utilization, and product formation has been shown to be 1:1:1 in some mixed-function oxidases containing P-450 (*37, 39*). Since considerable endogenous NADPH oxidation occurs in the absence of substrates, the stoichiometry for NADPH utilization was obtained by calculating the difference in the amount of NADPH used in the presence and absence of substrates. On addition of substrate, a rapid disappearance of NADPH occurs, an indication that there should be little autooxidation of P-450-Fe(II) in the presence of substrate (RH) or if the rate constant k_2 is much greater than k_1.



Therefore, if the above scheme is operative, the stoichiometry of the reaction should be proportional to the total amount of NADPH consumed from the time of substrate addition rather than a result of a difference in the amount of NADPH used as proposed, since the amount of autooxidation in the presence of RH should be reduced to a negligible level.

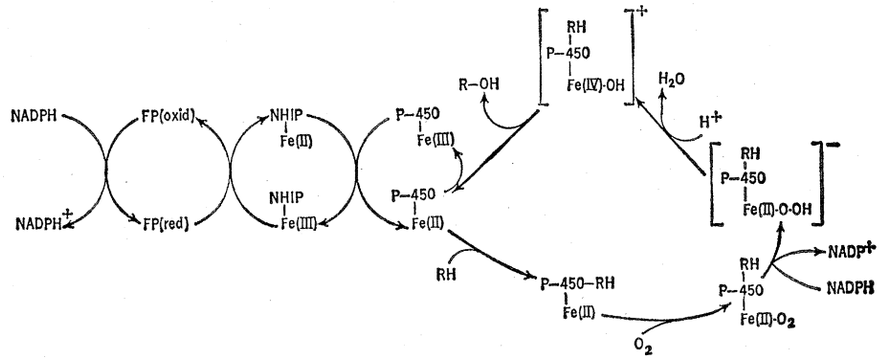
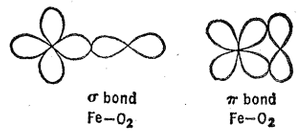


Fig. 2. Proposed mechanism of steroid hydroxylation. Abbreviations are: FP, adrenodoxin reductase; NHIP, adrenodoxin; RH, steroid; P-450, cytochrome P-450.

Another cogent objection to the above scheme is the postulation that the perferryl ion Fe(II)-O_2 is the so-called "active oxygen," for it is known that, in general, oxygen complexes of d_6-transition metals are kinetically stable toward oxidation of unactivated carbon-hydrogen bonds. This is because in Fe(II)-O_2, both (σ) and (π) bonding occur. In the σ bond, the ligand acts as a Lewis base and shares a pair of electrons with an empty e_g (dx^2-y^2) orbital; in the π bond, O_2 acts as a Lewis acid and accepts electrons from the filled T_{2g} orbital of the iron (d_{xy} orbital). This bonding and "backing" strengthens the metal-ligand and contributes to the unusual stability of the Fe(II)-O_2 complex.

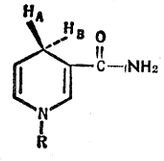


On the other hand, a much more reactive species would be the hydroperoxo complex of ferrous ion [Fe(II)-O-OH]$^-$. Within this complex, all the *p*-orbitals of oxygen are occupied, and "back bonding" by metal is not possible. This facilitates the rupture of the oxygen-oxygen bond and generates the ferryl ion complex Fe(II)-Ö: which is a highly reactive species (*40*).

The direct involvement of NADPH in the formation of [Fe(II)-O-OH]$^-$ in steroid hydroxylation had been proposed by Hayano (*41*). Although Kadis (*42*) reported that, when microsomes of sow ovaries were incubated with progesterone and tritiated NADPH, a substantial quantity (10 percent) of the tritium label appeared on the hydrogen of the 17-hydroxyl group. In view of the fact that the hydroxyl proton at C-17 undergoes rapid exchange with the medium (*43*), this result must be discounted. However, the Hayano mechanism is conceptually simpler and appears to satisfy all of the experimental data (Fig. 2). Inherent in this mechanism is the dual function of NADPH. Reduced NADP serves in an accessory capacity by keeping the autooxidizable P-450 in the reduced state by means of the NADPH-cytochrome P-450 reductase system, a sequence which is steroid independent. Second, NADPH is directly involved in the steroid hydroxylation reaction to generate the hydroperoxo complex [Fe(II)-O-OH]$^-$, as shown. It should be noted that there is no overall valence change of the P-450 on completion of one stoichiometric cycle of the reaction. Since the 11β-hydroxylation reaction proceeds with retention of configuration and obeys the rule of Bloom and Shull, it is represented as an electrophilic displacement reaction, where the "oxenoid" species (*44*) (OH^+) stereospecifically displaces the H on the steroid. The order of addition of oxygen, steroid, and NADPH to P-450 remains to be determined. Postulation of reduction of an iron-oxygen complex with formation of a reactive species is consistent with studies of the autooxidation of iron (II), whose second order dependence on ferrous ion concentration suggests rate-determining reduction of the complexes of ferrous ion and oxygen (*45*).

Experimental evidence in favor of the dual functional role of NADPH in 11β-hydroxylation of DOC has recently been obtained (*46*) and can be summarized as follows. (i) It is well known that the reduction of adrenodoxin by NADH can also be catalyzed by adrenodoxin reductase but at a con-

siderably slower rate than reduction by NADPH (*14*). By raising the concentration of adrenodoxin reductase, one can adjust the rate of reduction of adrenodoxin by NADH to a rate identical to that of NADPH. However, NADH still fails to support 11β-hydroxylation of DOC under the adjusted conditions. (ii) From initial velocity studies, a double reciprocal plot of $1/v$ (v, micromole of DOC hydroxylated per minute) against 1/NADPH concentration at varied fixed concentrations of DOC afforded a series of intersecting lines indicating that NADPH and DOC either combine with the same enzyme or with a different enzyme form; the enzyme forms are connected by reversible steps. If NADPH is only involved in an accessory capacity (for example, in the reduction of adrenodoxin), a series of parallel lines would have emerged. These conclusions are based on the assumption that the three proteins exist in a well-defined complex which can be considered to behave as a single enzyme. (iii) It was demonstrated that adrenodoxin reductase preferentially removes the H_B hydrogen of the NADPH molecule. The stereochemistry of tritiated NADPH oxidation was then examined with the complete 11β-hydroxylase components in the presence and absence of DOC. It was found that the DOC-dependent oxidation of tritiated NADPH involves the removal of the H_A hydrogen.



Summary

The existence of the transient [Fe(II)-O-OH]$^-$ species remains to be demonstrated, but in the absence of a better explanation for the DOC-dependent NADPH oxidation, a course which removes the H_A hydrogen, the suggestion that NADPH is involved in the reduction of Fe(II)-O_2 either directly or indirectly is most likely to be correct. Future experiments should be centered on the purification of the P-450 component and examination of the role of additional components, such as the heat-stable protein which enhances hydroxylation. Also, the stimulation of NADPH oxidation (*47*) by metopirone [2-methyl-1,2-bis(3-pyridyl)-1-propanone], a competitive inhibitor of 11β-hydroxylase, needs to be clarified. This article proposes that NADPH plays two distinct roles in the 11β-hydroxylase system of adrenal cortex mitochondria. It provides the reducing equivalents to keep the autooxidizable P-450-Fe(II) in the ferrous state of oxidation, a course which does not enter into the stoichiometry of the hydroxylation reaction. Second, it is directly involved in the formation of the so-called "active oxygen," [Fe(II)-O-OH]$^-$, a process which is steroid dependent. The other model presented is difficult to reconcile with some of the evidence discussed. It should be interesting to apply similar experimental approaches to examine other mixed-function oxidase systems containing P-450, to establish whether the proposed mechanism is widespread among external mixed-function oxidases (*2*).

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$$g = (h/\beta)\ (\nu/H)$$

where h is Planck's constant, β is the Bohr magneton, and ν and H are the frequency and magnetic field at resonance. See G. Palmer, *Meth. Enzymol.* **10**, 601 (1967).
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