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Studies on Steroid Monooxygenase from *Cylindrocarpon radicicola* ATCC 11011.[1] Oxygenative Lactonization of Androstenedione to Testololactone

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A steroid monooxygenase of *Cylindrocarpon radicicola* was found to catalyze oxygenative lactonization of 17-ketosteroid, androstenedione, to yield D-homo-17α-oxasteroid, testololactone, *i.e.*, the androstenedione monooxygenase reaction, in addition to catalyzing the progesterone monooxygenase reaction. The reaction product was identified by TLC, GLC, and mass spectrometry.

The oxygenation proceeded with unitary stoichiometry for 17-ketosteroid, NADPH, and molecular oxygen, indicating that it is a typical monooxygenase reaction of the external electron donor type. The enzyme catalyzed successively the side chain cleavage reaction of 17α-hydroxy-20-ketosteroid to produce its 17-keto derivative and the lactonization of the product.

The effects of pH and of the concentration of substrate steroids on the androstenedione monooxygenase reaction were different from those on the progesterone monooxygenase reaction. Progesterone is a strong and competitive inhibitor of the lactonization of 17-ketosteroids. The steroid monooxygenase is concluded to have the activities of both oxygenative esterification of 20-ketosteroids and oxygenative lactonization of 17-ketosteroids.

Several microorganisms metabolize progesterone to dehydrotestololactone through androstenedione and its oxidized, six-membered D-ring product, testololactone. The enzyme involved in this C_{19}-17-ketosteroid oxidation was studied by Prairie and Talalay with a crude extract from *Penicillium* sp. (*2*). They reported that androstenedione monooxygenase [EC 1.14.99.12] catalyzed the oxygenation of androstenedione to D-homo-17α-oxasteroid with concomitant consumption of NADPH and molecular oxygen. After that study, no attempt was made to characterize the enzyme for a long time. Miller suggested in a study of the fungal bioconversion of steroids that progesterone

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Abbreviations: dehydroepiandrostenedione, 5-androsten-3β-ol-17-one. Other abbreviations used are listed in the previous paper (*5*).

monooxygenase [EC 1.14.99.4] catalyzed the androstenedione monooxygenase reaction (*3*).

Cylindrocarpon radicicola ATCC 11011 is one of the fungi metabolizing progesterone to dehydrotestololactone (*4*). Steroid monooxygenase of this fungus, which has been purified and characterized as a typical flavoprotein enzyme (*5*), is an essential enzyme for oxidation of C_{21}-20-ketosteroids. The enzyme catalyzes a Baeyer-Villiger oxygenation of 20-ketosteroid to form the acetate ester of the 17-hydroxysteroid. In a study of the reactivity, it was discovered that the enzyme also has androstenedione monooxygenase activity. This paper describes the characterization of the androstenedione monooxygenase reaction catalyzed by the enzyme.

EXPERIMENTAL PROCEDURES

Enzyme Preparation—A steroid monooxygenase of *Cylindrocarpon radicicola* was purified as described in the preceding paper (*5*).

Buffer—In all of this work, unless otherwise noted, 0.03 M Tris-HCl buffer (pH 7.4) containing 0.1 mM Na-EDTA was used as the standard buffer, which is described as "Tris buffer" in this paper. As "phosphate buffer," 0.1 M potassium phosphate buffer (pH 6.5) containing 0.1 mM Na-EDTA was used.

Enzyme Assay—Progesterone monooxygenase activity was estimated by the reported procedures (*5*). The standard assay conditions for androstenedione monooxygenase activity are as follows: the activity was assayed by determining the rate of production of [^{3}H]testololactone from [^{3}H]androstenedione. The assay mixture contained, in the final volume of 0.51 ml, 196 μM [^{3}H]androstenedione (10^4 cpm), 10^3 cpm [^{14}C]testololactone, 98 μM NADPH, a suitable amount of the enzyme, and 30 mM potassium phosphate buffer (pH 6.5). Other procedures were the same as for progesterone monooxygenase except that dehydrotestololactone, whose R_f value is nearly the same as that of testololactone, was used as a marker steroid on TLC.

One unit of enzyme catalyzed the formation of 1 nmol of product per min at 35°C and the specific activity was defined as units per mg protein.

The enzyme activities were also estimated by measuring substrate steroid-stimulated NADPH oxidation at 340 nm at 35°C in 95 mM potassium phosphate buffer (pH 6.5) with an appropriate amount of the monooxygenase. For specific experiments, the enzyme was assayed by following oxygen uptake at 30°C with a Clark oxygen electrode in 90 mM potassium phosphate buffer (pH 6.5) with a suitable amount of the enzyme in a total volume of 1.2 ml.

Other experimental procedures were the same as described in the preceding paper (*5*).

Materials—Steroids used were obtained from six commercial sources. All radioactive steroids were products of Amersham International, and were [4-^{14}C]testosterone, [1α,2α(n)-^{3}H]progesterone, [4-^{14}C]androstenedione, [1,2,6,7-^{3}H]androstenedione, [1α,2α(n)-^{3}H]-17α-hydroxyprogesterone, and [1α,2α(n)-^{3}H]-17α-hydroxypregnenolone. [4-^{14}C]-Testololactone was prepared from [^{14}C]androstenedione by means of the enzyme reaction on a large scale and purified by TLC on a silica gel plate. Testololactone was obtained from the Steroid Collection, Department of Chemistry, London University, London. Dehydrotestololactone was prepared from a culture of *Cylindrocarpon radicicola* by extraction with benzene and purified by preparative TLC on a silica gel plate. The steroid lactone was crystallized from cyclohexane. Elemental analysis gave C, 75.55; H, 8.17: calculated for $C_{19}H_{24}O_3$: C, 75.94; H, 8.05.

NADPH and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim and Oriental Yeast Co., respectively. All other chemicals were commercial products of reagent grade.

RESULTS

Oxygenation of Androstenedione—Steroid monooxygenase catalyzed NADPH-dependent oxygenation of androstenedione under aerobic conditions, and the product was detected as a polar compound by TLC analysis. The product was identified as testololactone, a D-homo-17α-oxasteroid by TLC, GLC, and mass spectrometry. Its R_f value is 0.39 in a solvent mixture of benzene, ethyl acetate, and methanol (66/33/1, v/v) and the retention time is 5.7 min in GLC on a 1 m OV-1 column at 235°C. Figure 1 shows the mass spectrum of the product; the molecular ion (M^+/e) appeared at 302, the same position as that of the

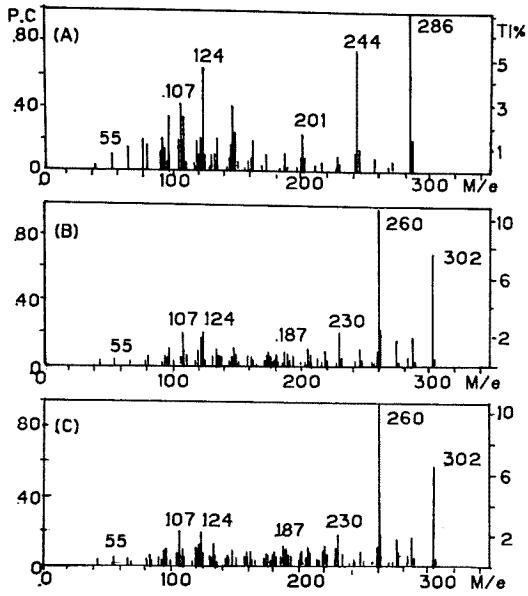

Fig. 1. Mass-spectral analysis of the reaction product. For the analysis of the reaction product, the enzyme reaction was carried out on a large scale. The system consisted of 210 μg of the oxygenase, 1.9 mM androstenedione, 0.84 mM NADPH, 10.5 mM $MgSO_4$, 3 mM glucose-6-phosphate-K, 20 μg of glucose-6-phosphate dehydrogenase and 25 mM Tris-HCl buffer, pH 7.4, in a total volume of 4.75 ml. The reaction was performed for 3 h at 35°C. Steroids were extracted with 3 ml of ethyl acetate and chromatographed on a preparative silica gel thin layer plate in benzene/ethyl acetate/methanol (66 : 33 : 1, v/v). The reaction product, testololactone, was eluted from the silica gel with methanol and recrystallized from *n*-hexane. The analysis was done on a Hitachi model M-80 mass spectrometer equipped with a data processing apparatus. (A) The substrate for the reaction, androstenedione, (B) the product of the steroid monooxygenase reaction, and (C) authentic sample of testololactone.

authentic compound, testololactone, and was 16 M^+/e units higher than that of androstenedione. The fragmentation pattern of the product was the same as that of testololactone (Fig. 1, B and C). It was concluded that the product of the monooxygenase reaction is testololactone, and insertion of one oxygen atom into the C-C bond between C13 and C17 occurs to form the steroid-lactone.

Properties of Androstenedione Monooxygenase Reaction—The enzyme reaction was linear with respect to the incubation time, and the rate was proportional to the amount of added enzyme up to 3 nmol of testololactone formed per min under the standard assay conditions. It was about 27 mol of testololactone formed/min/mol enzyme at 35°C. No activity of androstenedione monooxygenase in the crude extract from *C. radicicola* could be observed by any of several methods. The enzyme activity may be inhibited strongly by some endogenous metabolite(s) in the preparation. In contrast, the activity of progesterone monooxygenase was detected in the crude extract (*5*). The lactonization activity could be measured with the preparation eluted from the first DEAE-cellulose column in the purification of the enzyme.

Stoichiometry of the Androstenedione Monooxygenase Reaction—Figure 2, A and B, shows the stoichiometry of oxygenative lactonization of androstenedione; the amount of NADPH oxidized in the reaction was equal to the amount of product steroid (Fig. 2A) and also to the amount of molecular oxygen consumed (Fig. 2B), indicating that the reaction proceeds with the stoichiometric relation of a monooxygenase reaction.

Effect of pH—The maximum activity of the androstenedione monooxygenase reaction was observed at pH 6.5 in 50 mM potassium phosphate

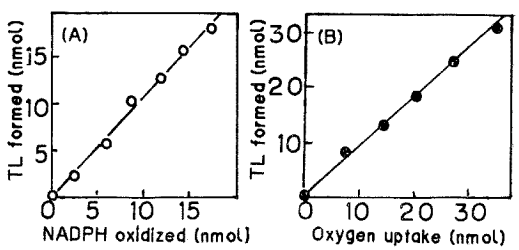

Fig. 2. Relations among the consumptions of NADPH and molecular oxygen and the production of testololactone in the steroid monooxygenase reaction. (A) NADPH oxidation and testololactone (TL) formation. The reaction was carried out in the standard assay system except that the concentration of NADPH was 32 μM. The consumption of NADPH and formation of testololactone were determined as described elsewhere (*2*). (B) Molecular oxygen consumption and testololactone (TL) formation. The reaction was carried out in a system containing 220 μM [^{3}H]androstenedione, 170 μM NADPH and an appropriate amount of the purified enzyme in a total volume of 1.2 ml of 0.1 M potassium phosphate buffer, pH 6.5. Oxygen consumption was monitored with an oxygraph at 35°C.

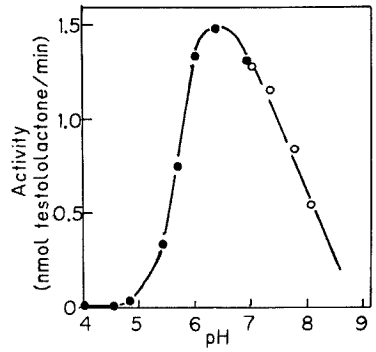

Fig. 3. Effect of pH on the androstenedione monooxygenase reaction of steroid monooxygenase. The reactions were carried out in a total volume of 0.55 ml of 0.05 M citric acid–0.1 M sodium monohydrogen phosphate buffer (pH 3.95–8.1) (●) or 0.03 M Tris-HCl buffer (pH 7.0–9.4) (○) at 35°C for 20 min.

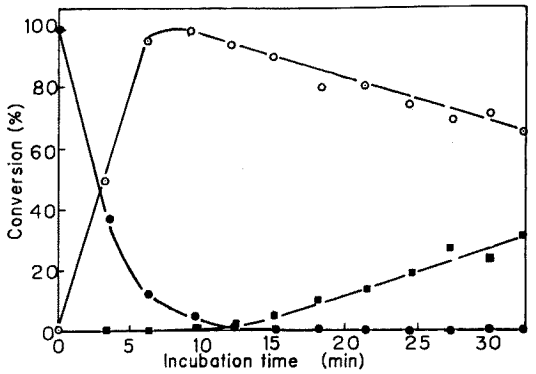

Fig. 4. Successive production of testololactone from 17α-hydroxyprogesterone by steroid monooxygenase. The reaction system consisted of 20 μM [^{3}H]17α-hydroxyprogesterone, 600 μM NADPH and 1.3 μg of enzyme in 0.5 ml of 0.03 M Tris-HCl buffer, pH 7.4. After incubation for the indicated time, the reaction was terminated and the amounts of substrate and product steroids were determined. ●, 17α-hydroxyprogesterone; ○, androstenedione; and ■, testololactone.

buffer (Fig. 3). This is different from the optimum pH for the progesterone monooxygenase reaction (pH 7.8) (*5*). The activity ratio between progesterone monooxygenase and androstenedione monooxygenase at the respective optimum pH values was about 3 under the standard assay conditions.

Oxygenation of 17α-Hydroxyprogesterone—The monooxygenase catalyzed overall conversion of 17α-hydroxyprogesterone into testololactone. This enzyme first cleaved the side chain of the substrate ketosteroid without the aid of esterase, and then produced androstenedione (*5*). As shown in Fig. 4, the substrate 17α-hydroxyprogesterone was consumed rapidly with the accumulation of androstenedione in the first phase, during which time testololactone was not produced. After the cleavage reaction, the oxygenative lactonization of the product proceeded at a lower rate.

Oxidation of NADPH during the reaction was biphasic; there was a rapid initial phase for about 2 min with the apparent rate constant $k' = 1 \times 10^{-2}\ s^{-1}/\mu g$ enzyme, followed by a slower and longer phase from about 20 min with $k' = 6.8 \times 10^{-4}\ s^{-1}/\mu g$ enzyme in the presence of 4 nmol of the substrate steroid. The former may represent the oxygenative esterification and the latter the oxygenative lactonization. Kinetic studies of the rates of oxygenation of steroid and NADPH revealed that the two reactions proceed successively and the apparent rate constant of the first reaction is about 15 times larger than that of the second reaction.

Kinetic Study—The androstenedione monooxygenase reaction was studied kinetically in detail. Lineweaver-Burk plots with various concentrations of androstenedione and NADPH and with a fixed concentration of molecular oxygen are shown in Fig. 5, A and B. When androstenedione was the variable substrate and NADPH was the fixed variable substrate, the lines converged to a common point on the x-coordinate at $-2.5 \times 10^{-2}\ \mu M^{-1}$ (Fig. 5A). When NADPH was the variable substrate and the steroid was the fixed variable substrate, the plots intersected at a common point above the x-coordinate at $-3.4 \times 10^{-1}\ \mu M^{-1}$ (Fig. 5B). The intercept replots of these plots, the secondary plots, are shown in Fig. 5, C and D, from which the K_m values for androstenedione and for NADPH were estimated to be 40 μM and 2 μM, respectively. The K_m value for androstenedione is larger by two orders of magnitude than that of progesterone (*5*).

Effect of Progesterone on the Androstenedione Monooxygenase Reaction—When a substrate-level concentration of progesterone was added to the assay system of the monooxygenase reaction, all the activity for lactonization of androstenedione would be inhibited until the 20-ketosteroid was

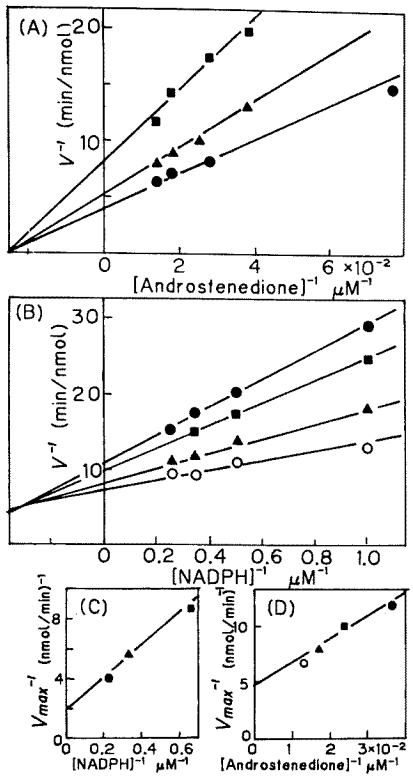

Fig. 5. Steroid monooxygenase activity as a function of androstenedione concentration at fixed concentrations of NADPH (A) or of NADPH concentrations at fixed concentrations of androstenedione (AD) (B). The assay system contained 2 μg of enzyme and variable substrate as indicated in a total volume of 1.0 ml of 0.1 M potassium phosphate buffer, pH 6.5. Reactions were initiated by addition of NADPH, and the velocity was determined by measuring the amounts of testololactone formed after incubation for 30 min (A) or 20 min (B) at 35°C. In (A), the concentrations of NADPH were 1.5 μM (■), 3.0 μM (▲), and 4.5 μM (●). In (B), the concentrations of androstenedione were 28 μM (●), 42 μM (■), 56 μM (▲), and 70 μM (○). (C) shows the secondary plots of the reciprocal of the apparent maximum velocity *versus* the reciprocal of the fixed substrate concentration from (A). (D) is that from (B).

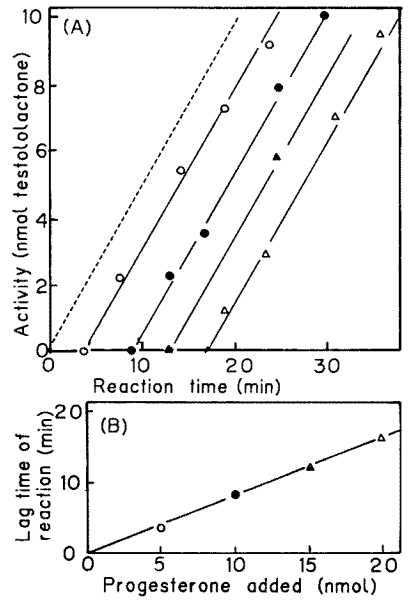

Fig. 6. Effect of progesterone on the oxygenative lactonization of androstenedione by steroid monooxygenase. (A) Time course of the reaction in the presence of various concentrations of progesterone. (B) Replots of the lag time in (A) against the amount of added progesterone. The amounts of added progesterone were 0 nmol (----), 5 nmol (○), 10 nmol (●), 15 nmol (▲), and 20 nmol (△). Other conditions were the same as the standard assay conditions.

esterified by the enzyme (Fig. 6A). The length of the lag time of lactonization in the figure was proportional to the amount of added progesterone (Fig. 6B). After the lag period, the lactonization reaction proceeded normally at the same rate as in a control assay mixture. In contrast, androstenedione was required at one hundred times the molar concentration of progesterone for half-inhibition of the esterification of progesterone.

Inhibition by Other Steroids—Pregnenolone and other C_{21}-20-ketosteroids, which are the substrates for oxygenative esterification of the enzyme (*5*), strongly inhibited the lactonization of androstenedione. The product of the progesterone monooxygenase reaction, testosterone acetate, as well as its esterase-hydrolyzed product, testosterone, competitively inhibited the oxygenative lactonization. 5-Pregnene-3β,20α-diol, which was not a substrate of this enzyme, similarly inhibited the lactonization. The estimated kinetic values are summarized in Table I.

Two Substrate Studies—The results shown in Fig. 5 suggest a sequential addition of two substrates to the enzyme in the reaction (*6*, *7*). An attempt to determine the order of addition of the substrates was made by using dehydroepiandrosterone as an alternate substrate (*8*). This 17-

TABLE I. Kinetic values of steroid monooxygenase.

	Androstenedione monooxygenase reaction	Progesterone monooxygenase reaction
K_m	(μM)	(μM)
Androstenedione	40	—
Progesterone	—	0.4
NADPH	2	4.3
Oxygen	n.d.[a]	70
K_I	(μM)	(μM)
Progesterone	< 1	—
Pregnenolone	< 1	—
Androstenedione	—	30
Dehydroepiandrosterone	17	30
Testosterone	50	n.d.[a]
Testosterone acetate	45	n.d.
5-Pregnene-3β,20α-diol	150	n.d.

[a] n.d., not determined.

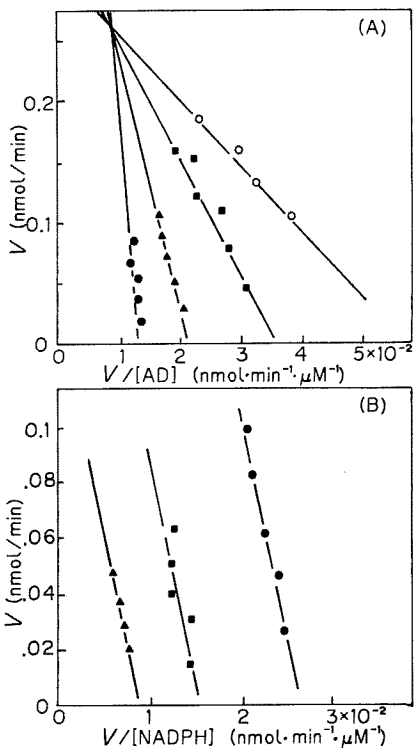

Fig. 7

ketosteroid was oxygenated by the enzyme with the consumption of NADPH to produce D-homo-17α-oxa-androst-5-en-3β-ol-17-one, which was identified by mass spectrometric analysis. The monooxygenase reactions were assayed at various concentrations of [^{3}H]androstenedione in the presence of dehydroepiandrosterone, as a fixed variable substrate, and at a fixed concentration of NADPH. The data were used to prepare plots of velocity (v) *versus* v/(concentration of androstenedione) (Fig. 7A), which gave the lines intersecting at a

Fig. 7. Effect of dehydroepiandrosterone on the androstenedione monooxygenase reaction. (A) The velocity (v) was determined in the presence of 0 μM (○), 13.5 μM (■), 33.8 μM (▲), or 67.6 μM (●) dehydroepiandrosterone. The concentration of NADPH was 125 μM and the concentration of androstenedione (AD) was varied as indicated. Other conditions were the same as those of the standard assay system. (B) The velocity (v) was determined in the presence of various concentrations of NADPH. The fixed concentration of androstenedione was 85 μM and the concentrations of dehydroepiandrosterone were 0 μM (●), 25 μM (■), and 50 μM (▲). The concentration of NADPH was varied. The total volume of the assay system was 1.0 ml with 0.1 M potassium phosphate buffer, pH 6.5.

common point with a v-value of 0.265 nmol/min. When NADPH was a variable substrate in the assay system, the resulting plots gave a set of parallel lines (Fig. 7B). These results suggest that the substrate 17-ketosteroid interacts first with the enzyme, and then NADPH does.

DISCUSSION

The androstenedione monooxygenase reaction, described by Prairie and Talalay (*2*), is a peculiar biological oxidation of steroids: an oxygen atom is inserted into the D-ring of the substrate steroid, producing a D-homo-17α-oxa-steroid. In spite of the interesting nature of the reaction, the chemical and enzymic characters of the enzyme have not been established. A study on the bioconversion of steroids suggested that a fungal progesterone monooxygenase, which was inducible, also acted as an androstenedione monooxygenase in the metabolic pathway of steroids (*3*). Progesterone monooxygenase catalyzes oxygenative esterification of progesterone to form testosterone acetate (*9*). In the present study, the enzyme was termed steroid monooxygenase on the basis of its catalytic properties.

Steroid monooxygenase of *Cylindrocarpon radicicola* has been purified to homogeneity and its molecular and catalytic properties characterized (*5*). The purified enzyme preparation also catalyzed the oxygenation of androstenedione with consumption of NADPH and molecular oxygen: this is the androstenedione monooxygenase reaction described above. Thus, it is established for the first time that the monooxygenase is a bifunctional enzyme. It catalyzes oxygenative lactonization of 17-ketosteroids and oxygenative esterification of 20-ketosteroids, which are biological Baeyer-Villiger type oxidations. Analogous enzymic reactions were reported with camphor monooxygenase (*10*), ketone monooxygenase (*11*), and cyclohexanone and cyclopentanone monooxygenases (*12*, *13*).

The results of mass spectral analysis of a lactonization product indicate that the oxygen insertion occurs only at the linkage of C13 and C17 on the D-ring of 17-ketosteroid to form a 17α-oxa compound, and not at the linkage of C16 and C17. The enzyme does not attack the 3-carbonyl carbon on the A-ring of androstenedione or the 11-carbonyl carbon on the C-ring of cortisone.

Unitary stoichiometry of the lactonization of 17-ketosteroid and also the esterification of 20-ketosteroid (*5*) for NADPH and molecular oxygen indicates the enzyme to be a typical monooxygenase of the external electron donor type. In both types of oxygenation, the enzyme displayed low K_m values for 20-ketosteroids in contrast to that for 17-ketosteroid, which was of the order of 10^{-5} M. Therefore, oxygenation of 17α-hydroxyprogesterone proceeded mainly in the initial phase with accumulation of the product, androstenedione, and after completion of this reaction, the second reaction, lactonization of androstenedione, occurred.

The kinetic study of the steady-state monooxygenase reaction suggested that the enzyme reacted sequentially with substrate steroid and electron donor NADPH, like several flavoprotein monooxygenases (*14–17*). Though some attempts were made to determine the order of binding in this study, further work is required to reach a conclusion.

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