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Influence of the Structure of the A and B Rings of Various 20-Oxo-steroids on Their Interaction with 20β-Hydroxysteroid Dehydrogenase

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In order to investigate the functional role of the region around the A and B rings of 20-oxo-steroids in the interaction with the binding site of 20β-hydroxysteroid dehydrogenase, kinetic studies were made using 28 kinds of steroids which differed in the nature of the substituent, and in the shape and electronic character of the region around the A and B rings. Structural changes in the A ring of steroids, such as reduction of pregn-4-ene derivatives to the corresponding 5α- or 5β-pregnane derivatives, introduction of a further double bond into the A ring, and change of the 5α-series to 5β-series or change in the configuration and size of the substituent at C-3 caused only small changes in the apparent K_m and V_{max} values. It is suggested that the region around the A ring is of little significance in the interactions between a steroid molecule and the enzyme. It seems unlikely that the electronic character and shape of the B ring affect the steroid-enzyme interaction, since introduction of a C-5/C-6 double bond scarcely changed the kinetic constants. Neither a nonpolar (methyl group) nor a polar (hydroxyl group) substituent at the C-6 α-position (equatorial) produced any appreciable changes in the kinetic constants. Further, a 6-methyl group and a 9α-fluoro group (axial) did not cause any marked changes in the kinetic constants.

On the other hand, introduction of a hydroxyl group at the C-6 β-position (axial) or removal of the 10β-methyl group caused a marked increase in the apparent K_m.

These findings suggest that recognition of the steroid moiety by the enzyme may involve the 10β-methyl group and the region around the β-side of the B ring. The nature of the interaction of these regions of the steroid with the enzyme may be hydrophobic, since a polar 6β-hydroxyl group had a marked repulsive effect on the steroid binding site of the enzyme, while a 10β-methyl group increased the binding affinity of the steroid.

Keywords——20β-hydroxysteroid dehydrogenase; 20-oxo-steroid; steroid; kinetic constants; interaction of steroid with enzyme; K_m and V_{max} for 20-oxo-steroids; steroid recognition by enzyme

Specific interactions between steroids and certain proteins are thought to be of prime importance in the biological action of steroids. It is therefore of general interest to identify the features in the molecular structure of steroids upon which interactions with functional proteins depend.

In our attempt to elucidate the basic mechanism by which a functional protein can interact specifically with a lipoid steroid, 20β-hydroxysteroid dehydrogenase [EC 1.1.1.53] from *Streptomyces hydrogenans* was chosen as a model protein, because this enzyme is available as a pure crystalline enzyme[1] in sufficient quantity for detailed comparative studies of steroid–protein interactions with various substituted steroids.

Our previous study on the substrate specificity of this enzyme revealed that the presence, nature and size of a substituent around the reacting 20-oxo group of steroids played a decisive role in the utilization of steroids by the enzyme, while structural changes in other parts of the steroid molecule including the A, B or C ring caused various changes in the efficiency of the utilization without entire loss of substrate reactivity towards the enzyme.[2] This suggests that the region around the C-17β-side chain, centering on the 20-oxo group, may be mainly involved in the catalytic interaction, and the ring moieties of the steroids may be main-

ly involved in the binding interaction with the enzyme. It was also suggested that the coenzyme (NADH) may be situated near C-16 of the steroid molecule in the steroid-coenzyme-enzyme ternary complex in the catalytic process.[2] Further kinetic study on the role of the regions around C-17 and C-21 suggested that the conformational relationships among substituents at C-17α, 20 and 21, and the configurational relationship between the plane of the steroid ring (or the D ring) and the C-17β-side chain might be important in steroid recognition by the catalytic site of the enzyme.[3] On the other hand, White and Jeffery deduced from a kinetic study using 6 substrates that the A ring of the steroid probably lies towards an extremity of the steroid-binding site of the enzyme.[4] They also suggested that interactions which are important for substrate function normally occur between the region around the B or C ring of the steroid and the enzyme.[5]

The present work was undertaken to elucidate more precisely the role of the regions around the A and B rings in the interaction of steroids with the enzyme. Kinetic measurements were made with 28 kinds of 20-oxo-steroids which differed in the nature of substituent, the shape, and the electronic character in the region around the A and B rings.

Experimental

Materials——Most of the steroids used were purchased from Sigma Chemical Co., E. Merck AG, or Fluka AG. Pregn-4-ene-3,20-dione, 11β,17,21-trihydroxypregn-4-ene-3,20-dione, and 11β,17,21-trihydroxypregna-1,4-diene-3,20-dione were standard substances from the National Institute of Hygienic Sciences, Tokyo. 9-Fluoro-11β,17-dihydroxy-6α-methylpregna-1,4-diene-3,20-dione and 17-hydroxy-19-norpregn-4-ene-3,20-dione were kindly supplied by Kowa Co., Ltd., Tokyo and Nihon Schering Co., Ltd., Osaka, respectively. The compound number used for each of the steroids is the same as that in the previous paper.[2] NADH was purchased from Sigma Chemical Co. and Oriental Yeast Co., Ltd., Tokyo. 20β-Hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* was obtained from Boehringer Mannheim GmbH, and its purity was checked as described previously.[2]

Concentrations of the Enzyme, NADH, and Steroids, and Steroid Solubility——These were determined as described in the previous paper.[2]

Assay of 20β-Hydroxysteroid Dehydrogenase——The enzyme activity was assayed at 25° by measuring the decrease in absorption of NADH at 340 nm under the conditions described in the previous paper,[2] except that the concentration of steroids was as indicated in Table I. The enzyme solution was made up freshly by dissolving aliquots of stock suspension (5 mg/ml in 2.2 M ammonium sulfate) in 5 mM sodium phosphate buffer (pH 7.0) containing 20% glycerol.[6] The amount of enzyme used was chosen so that the reaction was about 10—20% complete in about 1—3 min.[2] The initial rate of reaction was determined by fitting a straight line to the part of the curve corresponding to the first 10—20% of the reaction.

Kinetic Measurements——In principle, the initial rate determination of each steroid was carried out in duplicate at ten steroid concentrations ranging from appreciably below to appreciably above the predetermined apparent K_m value. However, in several steroid substrates, concentrations outside the range of their K_m had to be used because of their reactivity or their solubility. Linear regressions of the reciprocal of the initial reaction rate against the reciprocal of the substrate concentration were calculated by using the weighting procedure of Wilkinson.[7] The apparent K_m and apparent V_{max} were the reciprocals of the intercepts of these regression lines with the ordinate and abscissa, respectively.[8]

Standard errors of the apparent K_m and apparent V_{max} were estimated by Wilkinson's methods.[7] These values were calculated with a computer using the programs of Cleland.[9]

Results

Kinetic Constants for Various Steroid Substrates

The kinetic constants, the apparent K_m and apparent V_{max}, for various 20-oxo-steroid substrates are shown in Table I.

The efficiency of utilization of each steroid substrate is represented by the value of II, which is the ratio of the apparent K_m to the apparent V_{max} for the steroid.[4] Higher values of II correspond to decreasing efficiency of utilization of steroids.[4]

Table I. Kinetic Constants for Reduction of the 20-Oxo Group of Various Steroids by 20β-Hydroxysteroid Dehydrogenase from *Streptomyces hydrogenans*

Compd. No.[a]	Steroids	Concentration (μM)	V_{max} ± SE (μmol/min/mg)	K_m ± SE (μM)	Π (K_m/V_{max})
IA-1	Pregn-4-ene-3,20-dione	3.33—100	23.5 ±0.2	4.49±0.14	0.19
IA-2	17-Hydroxypregn-4-ene-3,20-dione	3.33—100	9.86±0.09	4.15±0.15	0.42
IA-3	Pregn-4-ene-3,11,20-trione	80.0—400	96.7 ±1.5	148 ±6	1.53
IA-4	17-Hydroxypregn-4-ene-3,11,20-trione	20.0—200	52.2 ±0.8	52.9 ±2.1	1.01
IA-5	17-Hydroxy-6α-methylpregn-4-ene-3,20-dione	3.33—50.0	4.26±0.04	2.32±0.01	0.54
IA-10	Pregna-4,16-diene-3,20-dione	13.3—133	0.83±0.09	191 ±28	230
IA-11	6α-Methylpregna-4,16-diene-3,20-dione	6.67—80.0	0.99±0.13	238 ±39	240
IA-16	17-Hydroxy-19-norpregn-4-ene-3,20-dione	14.8—165	41.7 ±0.8	32.5 ±1.6	0.78
IB-4	17,21-Dihydroxypregn-4-ene-3,11,20-trione	33.3—200	21.4 ±0.2	83.5 ±1.8	3.90
IB-5	6β,17,21-Trihydroxypregn-4-ene-3,11,20-trione	33.3—200	7.03±0.84	730 ±102	104
IB-7	11β,17,21-Trihydroxypregn-4-ene-3,20-dione	100 —400	3.42±0.08	317 ±12	92.7
IB-13	9-Fluoro-11β,17,21-trihydroxypregn-4-ene-3,20-dione	50.0—300	3.29±0.05	163 ±5	49.5
IIA-2	3β-Hydroxy-5α-pregnan-20-one	3.33—23.3	9.61±0.10	2.29±0.12	0.24
IIA-3	3β-Acetyloxy-5α-pregnan-20-one	3.33—13.3	4.73±0.07	0.85±0.10	0.18
IIA-4	5α-Pregnane-3,11,20-trione	33.3—200	72.6 ±2.3	217 ±11	3.00
IIB-1	17,21-Dihydroxy-5α-pregnane-3,11,20-trione	50.0—300	16.3 ±0.3	167 ±6	10.2
IIIA-1	5β-Pregnane-3,20-dione	4.00—100	10.9 ±0.1	6.97±0.26	0.64
IIIA-2	3α-Hydroxy-5β-pregnan-20-one	3.33—50.0	6.20±0.14	3.10±0.29	0.50
IIIA-3	3β-Hydroxy-5β-pregnan-20-one	4.00—66.7	6.06±0.09	4.45±0.27	0.73
IIIA-4	3α,6α-Dihydroxy-5β-pregnan-20-one	4.00—100	7.00±0.11	7.45±0.42	1.06
IVA-1	3β-Hydroxypregn-5-en-20-one	3.33—33.3	7.63±0.08	2.90±0.14	0.38
IVA-2	3β-Hydroxy-6-methylpregn-5-en-20-one	3.33—50.0	6.22±0.07	1.84±0.11	0.30
IVA-3	3β,17-Dihydroxypregn-5-en-20-one	3.33—33.3	5.70±0.11	2.22±0.23	0.39
IVA-4	3β,17-Dihydroxy-6-methylpregn-5-en-20-one	3.33—50.0	4.57±0.04	2.01±0.11	0.44
VB-1	17,21-Dihydroxypregna-1,4-diene-3,11,20-trione	33.3—200	25.6 ±0.5	70.2 ±3.5	2.74
VB-2	11β,17,21-Trihydroxypregna-1,4-diene-3,20-dione	100 —400	3.15±0.13	391 ±27	124
VB-3	11β,17,21-Trihydroxy-6α-methylpregna-1,4-diene-3,20-dione	80.0—400	3.84±0.13	240 ±16	62.5
VB-5	9-Fluoro-11β,17-dihydroxy-6α-methylpregna-1,4-diene-3,20-dione	20.0—100	8.81±0.25	57.8 ±3.2	6.56

a) All of the compound numbers and the classification are those used in our previous paper.[2]

Effect of Structural Changes in the A Ring and A/B Ring Junction on the Kinetic Constants

When the A ring of pregn-4-ene-3,11,20-trione (IA-3) and 17,21-dihydroxypregn-4-ene-3,11,20-trione (IB-4) was reduced to provide the corresponding 5α-pregnane derivatives, *i.e.*, 5α-pregnane-3,11,20-trione (IIA-4) and 17,21-dihydroxy-5α-pregnane-3,11,20-trione (IIB-1), respectively, changes in the kinetic constants were only small as shown in Table II-a.

Also, reduction of IA-1 to 5β-pregnane-3,20-dione (IIIA-1), though it is accompanied by a change of the A/B ring junction of the steroid skeleton, produced only a slight decrease in the apparent V_{max} and a slight increase in the apparent K_m (Table II-b).

Comparison of 3β-hydroxy-5β-pregnan-20-one (IIIA-3) with 3β-hydroxy-5α-pregnan-20-one (IIA-2), indicated that A/B *trans* (5α-series) form may be more suitable than A/B *cis* (5β-series) to interact effectively with the enzyme, since the 5β-series had a lower V_{max} value and higher K_m value than the 5α-series (Table II-c). However, the change of the A/B

Table II. Changes in Kinetic Constants upon Variation in the Structure of the A Ring

	Structural change	Compd. No.	Changes in kinetic constants (fold)		
			V_{max}	K_m	Π
a)	$\Delta^4 \rightarrow 5\alpha$	IA-3 → IIA-4	0.8	1.5	2.0
		IB-4 → IIB-1	0.8	2.0	2.6
b)	$\Delta^4 \rightarrow 5\beta$	IA-1 → IIIA-1	0.5	1.6	3.4
c)	$5\alpha \rightarrow 5\beta$	IIA-2 → IIIA-3	0.6	1.9	3.0
d)	$\Delta^4 \rightarrow \Delta^{1,4}$	IB-4 → VB-1	1.2	0.8	0.7
		IB-7 → VB-2	0.9	1.2	1.3
e)	3α-OH → 3β-OH	IIIA-2 → IIIA-3	1.0	1.4	1.5
f)	3β-OH → 3β-$OCOCH_3$	IIA-2 → IIA-3	0.5	0.4	0.8
g)	3=O → 3-OH	IIIA-1 → IIIA-2	0.6	0.4	0.8
		IIIA-1 → IIIA-3	0.6	0.6	1.1

ring junction resulted in only a small effect.

Introduction of a further double bond into the A ring of some pregn-4-ene derivatives (IB-4 to VB-1, IB-7 to VB-2) did not produce any marked effect on their apparent V_{max}, apparent K_m, or Π value (Table II-d), though the electronic character of the A ring and the spatial position of the substituent at C-3 in pregna-1,4-diene derivatives are considerably different from those of pregn-4-ene derivatives. It is suggested that there is neither appreciable steric restriction around position C-3 nor electrostatic requirement for the A ring in the interaction with the enzyme. This is consistent with the findings that 3α-hydroxy-5β-pregnan-20-one (IIIA-2) and the corresponding 3β-hydroxyl derivative (IIIA-3) have almost equal kinetic constants and that the esterification of the 3β-hydroxyl group to the 3β-acetyloxyl group (IIA-2 to IIA-3) decreased both the apparent V_{max} and K_m values to the extent of only about one-half, with no significant change in Π value (Table II-e, f). The change of the 3-oxo group of IIIA-1 to a 3α- or 3β-hydroxyl group (IIIA-1 to IIIA-2 or IIIA-3) also caused only a small change in the kinetic constants (Table II-g).

Effect of Structural Changes in the B Ring on the Kinetic Constants

Introduction of a C-5/C-6 double bond into IIA-2 (5α-pregnane series) caused little change in the apparent K_m and V_{max} values (Table III-a). Similarly, introduction of a C-5/C-6 double bond into a 5β-pregnane derivative (IIIA-3) resulted in only a slight decrease in the apparent K_m value and little increase in the apparent V_{max} value (Table III-b), though the

Table III. Changes in Kinetic Constants upon Variation in the Structure of the B Ring

	Structural change	Compd. No.	Changes in kinetic constants (fold)		
			V_{max}	K_m	Π
a)	$5\alpha \rightarrow \Delta^5$	IIA-2 → IVA-1	0.8	1.3	1.6
b)	$5\beta \rightarrow \Delta^5$	IIIA-3 → IVA-1	1.3	0.7	0.5
c)	6-H → 6α-CH_3	IA-2 → IA-5	0.4	0.6	1.3
		IA-10 → IA-11	1.2	1.3	1.0
		VB-2 → VB-3	1.2	0.6	0.5
d)	6-H → 6-CH_3	IVA-1 → IVA-2	0.8	0.6	0.8
		IVA-3 → IVA-4	0.8	0.9	1.1
e)	6-H → 6α-OH	IIIA-2 → IIIA-4	1.1	2.4	2.1
f)	6-H → 6β-OH	IB-4 → IB-5	0.3	8.7	26.7
g)	9-H → 9-F	IB-7 → IB-13	1.0	0.5	0.5
	(9-H → 9-F, 21-OH → 21-H)	VB-3 → VB-5	2.3	0.24	0.1
h)	10β-CH_3 → 10β-H	IA-2 → IA-16	4.2	7.8	1.9

modification is accompanied by transformation from the A/B-*cis* form to a more planar form as well as by a change in the electronic character in the region around the B ring.

Changes in the apparent V_{max}, K_m, and Π values caused by the introduction of a 6α-methyl group were slight and did not appear to be consistent (Table III-c). These changes appear to depend more strongly on other parts of the steroid structure than on the 6α-methyl group itself.

6-Methyl derivatives (IVA-2, IVA-4) of 3β-hydroxypregn-5-en-20-one (IVA-1) and 3β,17-dihydroxypregn-5-en-20-one (IVA-3), in which the 6-methyl group and the B ring are nearly coplanar, had kinetic constants approximately equivalent to those of their parent compounds (Table III-d). Therefore, the 6-methyl group as well as the 6α-methyl group may play no significant role in the interaction between the enzyme and steroid.

Introduction of a hydroxyl group at the C-6 α-position caused only a minor change in the kinetic constants (Table III-e).

On the other hand, introduction of a hydroxyl group at the C-6 β-position caused a marked change in the kinetic constants. When the kinetic constants of 17,21-dihydroxypregn-4-ene-3,11,20-trione (IB-4) are compared with those of its 6β-hydroxyl derivative(IB-5), the values for the latter were one-third as large for V_{max}, 8.7 times larger for K_m, and 26.7 times larger for Π compared with the former (Table III-f). It should be noted that compound IB-4 itself was one of the steroids having a relatively high K_m value (Table I) and the introduction of the 6β-hydroxyl group into this compound led to further enhancement, to give the highest K_m value observed among the steroids tested (Table I). Its K_m value was found to be about 163 times that of pregn-4-ene-3,20-dione (IA-1), while its V_{max} value was only about one-third of that of IA-1.

An example of the effect of introduction of a fluoro group into the C-9 α-position on the kinetic constants can be seen by comparing the results for 11β,17,21-trihydroxypregn-4-ene-3,20-dione (IB-7) and 9-fluoro-11β,17,21-trihydroxypregn-4-ene-3,20-dione (IB-13). The substitution caused a slight decrease in the apparent K_m value (to one-half), but no change in the apparent V_{max} value (Table III-g). Another example may be found by comparing 9-fluoro-11β,17-dihydroxy-6α-methylpregna-1,4-diene-3,20-dione (VB-5) with 11β,17,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione (VB-3), though this does not necessarily represent the net effect of the introduction of the 9-fluoro group. On changing the substrate from VB-3 to VB-5, the K_m and Π values decreased to one-fourth and one-tenth, respectively, and the V_{max} increased 2.3-fold (Table III-g). Substitution of a hydrogen for a hydroxyl group at C-21 involves structural changes, in addition to the introduction of a 9α-fluoro group, in going from VB-3 to VB-5. As an example of the characteristic effect of the substitution of a hydrogen for a hydroxyl group at C-21 on the kinetic constants, comparison of the kinetic data for 17,21-dihydroxypregn-4-ene-3,11,20-trione (IB-4) with those for 17-hydroxypregn-4-ene-3,11,20-trione (IA-4) may be useful (Table I). The change of substrate from IB-4 to IA-4 produced a 2.4-fold increase in the apparent V_{max} value and a decrease in the apparent K_m (to three-fifths) and in Π value (to one-fourth). The increase in the V_{max} value was similar in extent to that on going from VB-3 to VB-5, but the extent of the decrease in the K_m value as well as Π value in going from IB-4 to IA-4 was rather small compared to that from VB-3 to VB-5. These findings suggest that the introduction of a fluoro group at C-9 may not affect the catalytic process, and may have only a little effect on the binding process.

The change of substrate from 17-hydroxypregn-4-ene-3,20-dione (IA-2) to 17-hydroxy-19-norpregn-4-ene-3,20-dione (IA-16) produced a 4.2-fold increase in the apparent V_{max} value, a 7.8-fold increase in the apparent K_m value, and a 1.9-fold increase in the Π value (Table III-h). It is noteworthy that removal of the 10β-methyl group resulted in a significant increase in the apparent V_{max} value as well as in the K_m value, in contrast to the case of the introduction of a 6β-hydroxyl group, which resulted in a marked increase in the apparent K_m, but a decrease in the apparent V_{max}.

Discussion

It is suggested that the nature of the substituent on the A ring and the shape and electronic character of the region around the A ring may be of relatively little significance in the interaction between a steroid molecule and the enzyme, since changes in the kinetic constants with variation in the structure of the A ring of 20-oxo-steroids seem to be too small to be consistent with a significant role of this region in the enzyme-steroid interaction (Table II). This is consistent with the previous suggestion that the A ring probably lies towards an extremity of the substrate-binding site of the enzyme.[4]

The small changes in the kinetic constants on the introduction of a C-5/C-6 double bond suggest that there is little or no participation of the electronic character and shape of the B ring in the steroid-protein interaction. Neither a nonpolar (methyl group) nor a polar (hydroxyl group) substituent at the C-6 α-position (equatorial), or the 6-methyl group of various steroids, had any appreciable effect on the kinetic constants. Also, the findings that the introduction of a 9α-fluoro group (axial) did not cause any change in the V_{max} value and did not have any marked effect on the K_m value suggest that the enzyme may not interact with such a halogen group significantly. These results indicate that there may be neither a polar nor a hydrophobic binding site on the enzyme to interact with the near α-side of the B ring or, if there is, that it might be either very flexible or relatively unimportant.

It should be noted that the introduction of a 6β-hydroxyl group (axial) or removal of the 10β-methyl group (axial) caused a marked increase in the apparent K_m, though the former decreased the apparent V_{max} value and the latter caused a significant increase in the apparent V_{max}.

In molecular terms, this would mean that the introduction of a 6β-hydroxyl group or removal of the 10β-methyl group may have an unfavorable effect on the affinity of the steroid and the enzyme. In other words, the 6β-hydroxyl group may have a repulsive effect on the binding site of the enzyme, whereas the 10β-methyl group could be thought of as increasing the steroid-protein binding. The repulsive effect of the 6β-hydroxyl group may indirectly lead to slight departure of the position of the 20-oxo group from the optimum position for hydrogen transfer at the catalytic site of the enzyme, and thereby may result in a decrease in the efficiency of hydrogen transfer. The absence of the 10β-methyl group may indirectly bring about a somewhat greater flexibility in the interaction of the reacting 20-oxo group with the catalytic site of the enzyme to enhance the efficiency of the hydrogen transfer stage. This, together with acceleration of the leaving of the product steroid which lacks the 10β-methyl group, may be responsible for an increase in the apparent V_{max}.

It has been shown that the reaction mechanism of 20β-hydroxysteroid dehydrogenase is essentially an ordered Bi Bi mechanism, in which the enzyme first binds the coenzyme (k_1/k_2) and then the steroid (k_3/k_4), hydrogen is transferred (k_5/k_6), and the steroid product leaves the enzyme (k_7/k_8) followed by the coenzyme product (k_9/k_{10}).[10] Application of a steady-state kinetic treatment[11] to this ordered Bi Bi mechanism gives the apparent K_m and apparent V_{max} values in terms of the individual rate constants[5a] and these equations can be simplified as follows if it is assumed that 150 μM NADH (used at this fixed concentration throughout the present study) is a saturating concentration. This is supported by the findings that the apparent K_m value for NADH was about 2—4 μM for various 20-oxo-steroid substrates[2,4,5] and k_2/k_1 was 3.7—5.0 μM.[10a]

$$\text{Apparent } K_m \text{ for 20-oxo-steroid} = \frac{(k_4k_6+k_4k_7+k_5k_7)k_9}{(k_5k_7+k_5k_9+k_6k_9+k_7k_9)k_3} \quad (1)$$

$$\text{Apparent } V_{max} \text{ for 20-oxo-steroid} = \frac{[E]_{total} \times k_5k_7k_9}{(k_5k_7+k_5k_9+k_6k_9+k_7k_9)} \quad (2)$$

$$\Pi = \frac{K_m}{V_{max}} = \frac{k_4k_6 + k_4k_7 + k_5k_7}{k_3k_5k_7} \quad (3)$$

From Eqs. (1), (2) and (3), the changes of the apparent K_m, and apparent V_{max} and Π values obtained experimentally can be correlated with changes in the rate constants of each reaction step.

On this basis, if there is a decrease in k_3 (or an increase in k_4/k_3, which represents the dissociation constant of 20-oxo-steroid with the enzyme-NADH binary complex) and also probably in k_7 which describes the dissociation of steroid product from enzyme-NAD^+-alcohol ternary complex and seems to be closely related to the change in k_4/k_3, the apparent K_m for steroid will increase [Eq. (1)]. Similarly, inspection of Eq. (2) indicates that a considerable decrease in k_5, which is related to the efficiency of hydrogen transfer, causes a decrease in the apparent V_{max}; even when k_7 increases, a decrease in V_{max} can occur if the degree of the decrease in k_5 is sufficiently larger than that of the increase in k_7. Under conditions where the degree of the decrease in k_3 (or increase in k_4/k_3) as well as the decrease in k_5 is sufficiently larger than that of the increase in k_7, a relatively larger increase in Π value could arise [Eq. (3)]. These may correspond to the changes caused by the introduction of a 6β-hydroxyl group.

On the other hand, when k_4/k_3, k_7 and probably k_5 all increase, and also when the degree of the increase in k_4/k_3 (or decrease in k_3) is larger than those of k_7 and/or k_5, a significant increase in both the apparent K_m and V_{max} and a slight increase in Π value would be expected to occur. This may correspond to the removal of the 10β-methyl group.

At any rate, it is suggested that there may be significant steroid recognition by the enzyme around the β-side in the position 6 to 10 region. The nature of the interaction may be hydrophobic since the small and polar 6β-hydroxyl group had a marked repulsive effect, but a nonpolar 10β-methyl group increased the affinity to the binding site of the enzyme.

A similar finding was reported by White and Jeffery.[5a]

On the basis of these findings, it may be concluded that neither the A ring nor the B ring of pregnan-20-one derivatives is a major contributor to the binding process with the enzyme, but, in the steroid-enzyme complex, a hydrophobic region which forms some part of the binding site of the enzyme may be situated over the region of the β-side of the B ring and may interact with the 10β-methyl group.

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