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Structural Requirements in 20-Oxo-steroids for Interaction with the Binding Site of 20β-Hydroxysteroid Dehydrogenase

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In order to investigate the functional role of the region around the C and D rings in the interaction of steroids with the binding site of 20β-hydroxysteroid dehydrogenase, kinetic measurements were made for 34 kinds of steroids which differed in the nature of substituents, and in the shape and electronic character of the region around the C and D rings. Introduction of an oxo group at C-11 increased the apparent V_{max}, apparent K_m and Π (K_m/V_{max}) values 2- to 9-, 13- to 195-, and 2- to 30-fold, respectively. Introduction of a hydroxyl group at the C-11 β-position markedly increased the apparent K_m (58- to 119-fold) and Π (164- to 256-fold) values, but decreased the apparent V_{max} value to one-half to one-third. An 11α-hydroxyl group caused an increase in the apparent K_m value similar to that caused by an 11β-hydroxyl group, but the degree of decrease in the apparent V_{max} value was rather lower. Esterification of the 11α-hydroxyl group led to a decrease in the apparent K_m value (0.3-fold) without any significant change in the V_{max} value. It is suggested that a binding interaction may occur between the region around C-11 of the steroid and the enzyme-coenzyme complex; the interaction is probably hydrophobic in nature. A substituent at C-16 had an inhibitory effect on the hydrogen transfer stage since it resulted in loss of the substrate activity or a marked decrease in the V_{max} value (to about 1%) and an increase in the K_m value (23-fold). Introduction of a C-16/C-17 double bond, which caused a change in the configurational relationship between the 17β-side chain and the D ring, markedly decreased the apparent V_{max} value (to one-thirtieth) and increased the apparent K_m value (43-fold). Introduction of a hydroxyl group at C-18 had a marked effect on the kinetic constants, though the extent of the effect depended on the substituent at C-21. The steric and polar properties of the substituent at C-18 seem to be important factors in the interaction of the steroid with the binding site of the enzyme, and indirectly in that with the catalytic site. The features of the interaction between 20β-hydroxysteroid dehydrogenase and 20-oxo-steroids, as deduced from the results of the present and previous studies, are discussed.

Keywords——steroids; 20-oxo-steroids; 20β-hydroxysteroid dehydrogenase; binding site; kinetic constants; V_{max} and K_m for 20-oxo-steroids; interaction between the enzyme and steroid

We have already studied the basic mechanism involved in the interaction between 20β-hydroxysteroid dehydrogenase [EC 1.1.1.53] from *Streptomyces hydrogenans* and various 20-oxo-steroids.[1] Examination of substrate specificity suggested that the binding interaction with the enzyme may mainly involve the steroid ring, while the catalytic process involves the region around C-16—C-21, centering on C-20.[1a] Kinetic studies indicated that the coenzyme (NADH) may be located in the vicinity of C-16 in the ternary complex,[1a] that the orientation of the reacting 20-oxo group, as well as the size of substituents and the conformational relationships among substituents at C-17α, 20, and 21 play an important role in the interaction between 20-oxo-steroids and the catalytic site of the enzyme,[1b] and that the β-side of the B ring involving the 10β-methyl group may be recognized by the binding site of the enzyme whereas the A ring and the other part of the B ring may not.[1c] On the other hand, White and Jeffery suggested the importance of the region of C-11 (C-ring), in addition to the β-side of the B ring, for substrate binding.[2]

The present work was designed to elucidate more precisely the role of the regions around

the C and D rings in the interaction of 20-oxo-steroids with the enzyme. Kinetic constants obtained from 34 kinds of steroids, which differed in the nature of the substituents, and in the shape and electronic character in the region around the C and D rings, were evaluated in relation to the structural requirements in 20-oxo-steroids for interaction with the enzyme. Possible features of the interaction between the enzyme, coenzyme, and 20-oxo-steroid in the ternary complex system are deduced.

Experimental

Material——Most of the steroids were obtained 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 National Institute of Hygienic Sciences, Tokyo. The compound number for each of the steroids is the same as that in the previous paper.[1a] NADH was purchased from Sigma Chemical Co. and Oriental Yeast Co., Tokyo. 20β-Hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* was obtained in a crystalline form from Boehringer Mannheim GmbH. The purity of the enzyme was checked as described previously.[1a]

Concentrations of the Enzyme, NADH, and Steroids, and Steroid Solubility——These were determined as described in the previous paper.[1a]

Initial-Rate Determinations——These were made as described in the previous paper.[1a,c]

Determination of Apparent K_m and Apparent V_{max}——In principle, these were carried out as described in the previous paper.[1b,c] 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.[3] The apparent K_m and apparent V_{max} were the reciprocals of the intercepts of these regression lines on the ordinate and abscissa, respectively. Standard errors of the apparent K_m and apparent V_{max} were estimated by Wilkinson's method.[3] These values were calculated with a computer using the programs of Cleland.[4]

Interpretation of the Kinetic Constants——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}).[5] It is assumed that the mechanism of reaction is essentially the same for all the substrates used in the present study. Application of a steady-state kinetic treatment[6] to this ordered Bi Bi mechanism gives the apparent K_m and V_{max} values in terms of the individual rate constants.[2a] These equations can be simplified as follows, if it is assumed that 150 μM NADH (used at this concentration throughout the present study) is a saturating concentration. This view is supported by the findings that the apparent K_m value for NADH was about 2—4 μM for various 20-oxo-steroid substrates[1a,2,7] and that k_2/k_1 was 3.7—5.0 μM.[5a]

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

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

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

Π is a parameter representing the "efficiency of utilization" of the steroid substrates. A decrease in the efficiency of utilization of a steroid (decrease in k_3, k_5, and/or k_7) corresponds to a higher Π value.

Results and Discussion

Kinetic Constants for Various 20-Oxo-steroids

Table I shows the apparent K_m, apparent V_{max}, and Π for 34 kinds of 20-oxo-steroids which differ in the nature of substituents, and in the shape and electronic character of the region around the C and D rings.

Effect of the Introduction of an Oxo Group at C-11 on the Kinetic Constants

In general, the introduction of an oxo group at C-11 increased apparent V_{max}, apparent K_m and Π values 2- to 9-, 13- to 195-, and 2- to 30-fold, respectively (Table II-a), and the

magnitude of the increase in the apparent K_m value was greater in the case of 17-deoxy-steroids than in the case of 17-hydroxy-steroids. Changes of 17-hydroxypregn-4-ene-3,20-dione (IA-2) and 17,21-dihydroxypregn-4-ene-3,20-dione (IB-2) in the 17-hydroxypregn-4-ene series to the corresponding 11-oxo derivatives caused about 13- and 15-fold increases in the apparent K_m value, respectively, whereas changes of pregn-4-ene-3,20-dione (IA-1) and 21-hydroxy-

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

Compd. No.	Steroids	Concentration (μM)	Apparent V_{max} ± SE (μmol/min/mg)	Apparent 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-6	11α-Acetyloxypregn-4-ene-3,20-dione	50.0 —300	14.3 ±0.4	137 ±7	9.58
IA-7	11α-Hydroxypregn-4-ene-3,20-dione	50.0 —300	16.5 ±0.7	464 ±28	28.1
IA-8	11β-Hydroxypregn-4-ene-3,20-dione	50.0 —300	10.4 ±0.5	505 ±34	48.6
IA-9	18-Hydroxypregn-4-ene-3,20-dione	33.3 —200	8.9 ±1.6	1025 ±205	115
IA-10	Pregna-4,16-diene-3,20-dione	13.3 —133	0.83±0.09	191 ±28	230
IA-12	16α-Methylpregn-4-ene-3,20-dione	20.0 —200	0.25±0.01	105 ±7	420
IB-1	21-Hydroxypregn-4-ene-3,20-dione	4.00—100	3.72±0.04	8.14±0.34	2.19
IB-2	17,21-Dihydroxypregn-4-ene-3,20-dione	3.33—100	9.75±0.09	5.45±0.20	0.56
IB-3	21-Hydroxypregn-4-ene-3,11,20-trione	50.0 —300	12.0 ±0.5	253 ±17	21.1
IB-4	17,21-Dihydroxypregn-4-ene-3,11,20-trione	33.3 —200	21.4 ±0.21	83.5 ±1.8	3.90
IB-6	11β,21-Dihydroxypregn-4-ene-3,20-dione	100 —400	1.18±0.02	576 ±14	488
IB-7	11β,17,21-Trihydroxypregn-4-ene-3,20-dione	100 —400	3.42±0.08	317 ±12	92
IB-8	11β,21-Dihydroxy-3,20-dioxopregn-4-en-18-al	50.0 —200	0.03±0.00	246 ±50	8200
IB-14	18,21-Dihydroxypregn-4-ene-3,20-dione	120 —300	0.26±0.03	530 ±85	2040
IIA-4	5α-Pregnane-3,11,20-trione	33.3 —200	72.6 ±2.3	217 ±11	3.00
IIIA-2	3α-Hydroxy-5β-pregnan-20-one	3.33—50.0	6.20±0.14	3.10±0.29	0.50
IIIA-5	3α,17-Dihydroxy-5β-pregnan-20-one	4.00—66.7	3.57±0.05	1.78±0.16	0.50
IIIA-6	3α-Hydroxy-5β-pregnane-11,20-dione	26.7 —200	40.0 ±1.9	604 ±34	15.1
IIIA-7	3α,17-Dihydroxy-5β-pregnane-11,20-dione	33.3 —200	32.8 ±0.25	84.0 ±1.43	2.56
IIIA-8	3α,7α,12α-Trihydroxy-5β-pregnan-20-one	40.0 —200	0.65±0.09	506 ±91	778
IIIB-1	3α,17,21-Trihydroxy-5β-pregnan-20-one	3.33—66.7	4.38±0.10	3.28±0.34	0.75
IIIB-2	3α,17,21-Trihydroxy-5β-pregnane-11,20-dione	50.0 —300	7.99±0.20	121 ±6.8	15.1
IIIB-3	3α,11β,21-Trihydroxy-5β-pregnan-20-one	66.7 —200	0.21±0.02	353 ±42	1680
IIIB-4	3α,11β,17,21-Tetrahydroxy-5β-pregnan-20-one	66.7 —400	2.18±0.07	390 ±20	179
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

Table II. Changes in Kinetic Constants upon Variation of the Substituent at C-11

	Structural change	Compd. No.	Changes in kinetic constants (fold)		
			V_{max}	K_m	Π
a)	11-H → 11=O	IA-1 → IA-3	4.1	33	8.1
		IB-1 → IB-3	3.2	31	9.6
		IA-2 → IA-4	5.3	13	2.4
		IB-2 → IB-4	2.2	15	7.0
		IIIA-2 → IIIA-6	6.5	195	30
		IIIA-5 → IIIA-7	9.2	47	5.1
		IIIB-1 → IIIB-2	1.8	37	20
b)	11-H → 11β-OH	IA-1 → IA-8	0.44	112	256
		IB-1 → IB-6	0.32	71	223
		IB-2 → IB-7	0.35	58	164
		IIIB-1 → IIIB-4	0.50	119	239
c)	11-H → 11α-OH	IA-1 → IA-7	0.70	103	148
d)	11-H → 11α-$OCOCH_3$	IA-1 → IA-6	0.61	31	50
e)	11α-OH → 11α-$OCOCH_3$	IA-7 → IA-6	0.87	0.30	0.34

pregn-4-ene-3,20-dione (IB-1) in the pregn-4-ene series caused more marked increases (33- and 31-fold). Also, although introduction of a 11-oxo group into 3α-hydroxy-5β-pregnan-20-one (IIIA-2) in the 5β-pregnane series increased the apparent K_m about 195-fold, the same change in compounds of the 17-hydroxy-5β-pregnane series, such as 3α,17-dihydroxy-5β-pregnan-20-one (IIIA-5) and 3α,17,21-trihydroxy-5β-pregnan-20-one (IIIB-1), only resulted in increases of about 47- and 37-fold, respectively.

Inspection of Eq. (1), (2), and (3) indicates that an increase in the values of all three parameters may be ascribable to conditions where k_4/k_3 and k_7 increase, and the degree of the increase in k_4/k_3 is larger than that in k_7. Increase in k_5/k_6 may also occur, though the extent may be small.

In molecular terms, this would mean that the 11-oxo group may reduce the affinity of 20-oxo-steroids for the enzyme-NADH binary complex and also facilitate dissociation of the 20β-hydroxy-steroid as a product from the enzyme-NAD^+-alcohol ternary complex. The 17α-hydroxyl group may be able to compensate for the reduced affinity of the steroid owing to the presence of the 11-oxo group, as mentioned in our previous paper.[1b]

Effect of the Introduction of a Hydroxyl Group at C-11 on the Kinetic Constants

Introduction of a hydroxyl group at the C-11 β-position (axial) caused a marked increase in the apparent K_m values (58- to 119-fold) and Π value (164- to 256-fold), as shown in Table II-b. However, introduction of the 11β-hydroxyl group decreased the apparent V_{max} value to one-half to one-third, in contrast to the case of the 11-oxo group, which resulted in an increase in V_{max}. A marked increase in the apparent K_m value may be attributed to a significant increase in k_4/k_3, and a decrease in the apparent V_{max} value to a decrease in k_5 or k_7. However, k_7, which concerns the dissociation of the 20β-hydroxy-steroid from the enzyme-NAD^+-alcohol ternary complex, may be expected to increase somewhat under conditions where k_4/k_3 (which represents the dissociation constant of the 20-oxo-steroid and the enzyme-NADH binary complex) is increased markedly. If the degree of the increase in k_7 is sufficiently smaller than that of the increase in k_4/k_3 and/or decrease in k_5, an increase in Π value and a decrease in the apparent V_{max} could arise.

This suggests that the 11β-hydroxyl group (axial) may markedly weaken the binding interaction between the steroid substrate and enzyme-coenzyme complex, and may also have an unfavorable effect on the efficiency of hydrogen transfer.

As shown by the comparison of IA-1 with 11α-hydroxypregn-4-ene-3,20-dione (IA-7), the introduction of a hydroxyl group at the C-11 α-position (equatorial) resulted in an increase in the apparent K_m value (103-fold) similar to that caused by the 11β-hydroxyl group, but the degree of the decrease in the apparent V_{max} value was lower (Table II-c). It seems that the unfavorable effect of the 11β-hydroxyl group on the steroid-binary complex of enzyme-NADH interaction may be greater than that of the 11α-hydroxyl group.

It is noteworthy that upon introduction of an acetyloxyl group at the C-11 α-position (equatorial), the magnitude of the increase in the apparent K_m value became less (31-fold) than in the case of the 11α-hydroxyl group, while the change in the V_{max} value was not so marked (Table II-d). This means that esterification of the 11α-hydroxyl group led to the decrease in the apparent K_m value (to about one-third) without any significant change in V_{max} (Table II-e). Since an acetyloxyl group is more bulky but less polar than a hydroxyl group, it seems possible that the unfavorable effect of a C-11 hydroxyl group on the binding interaction between the steroid and enzyme-coenzyme binary complex may be ascribable more to polar nature than to steric factors.

We conclude that there may be some binding site on the enzyme that interacts with the steroid at around C-11, and that the nature of the interaction may be hydrophobic. The findings that the introduction of an oxo and a hydroxyl group at C-11 generally resulted in a significant increase in the apparent K_m, and that the apparent K_m values of 11β-hydroxyl derivatives were always higher than those of the corresponding 11-oxo derivatives may also indicate a repulsive effect of a polar group with respect to the binding site in the hydrophobic region of the enzyme, though the steric repulsive effect of the substituent may also be significant.

In spite of the common effect of an oxo and a hydroxyl group at C-11 on the binding interaction, an opposite effect on the apparent V_{max} value was found on the introduction of the 11-oxo group and 11-hydroxyl group; all 11-oxo derivatives had higher V_{max} values than the corresponding 11-deoxy derivatives, but all 11-hydroxyl derivatives had lower values (Table II). Similar findings have been reported by White and Jeffery,[2b] though only a few examples were given. To explain these phenomena they assumed that interactions important for the substrate function arose from the so-called hydrophobic forces between the generally hydrophobic C ring portion of the substrate and a hydrophobic region of the enzyme, but that when the substrate contained a polar substituent in this portion of the molecule, polar interactions with a polar moiety of the enzyme, which is a flexible part of the enzyme and undergoes a substrate-induced fit, could also be important. However, it is difficult to imagine how a polar 11-oxo group can bring about a marked increase in both K_m and V_{max}, while the more polar 11-hydroxyl group brought about a decrease in V_{max} and a marked increase in K_m. Thus, an alternative explanation seems to be more probable. On the basis of Eqs. (1) and (2), and the above discussion, it was assumed that the 11-oxo group caused a reduction in the binding affinity of steroids (increase in k_4/k_3) through polar and steric repulsion against the binding site of the enzyme, but that this could not modify the alignment of the steroid molecule to affect the interaction of the reacting 20-oxo group with the catalytic site of the enzyme unfavorably. However, after hydrogen transfer to the 20-oxo group, the resulting 20β-hydroxyl group (which may interact with the catalytic site in a somewhat different manner stereochemically compared with the 20-oxo group), cooperating with the 11-oxo group, may change the alignment of the steroid to reduce both the binding affinity of steroid (increase in k_7) and the reactivity toward the catalytic site of the enzyme (decrease in k_6); such a change in rate constants may be reflected as an increase in V_{max}. This is supported by the findings that the 11-oxo group in 20β-hydroxy derivatives generally causes a marked decrease in the reactivity of steroids with the enzyme.[8] On the other hand, polar and steric repulsion owing to the presence of the 11-hydroxyl group, especially at the β-position, may produce an alignment of steroid so unfavorable as to reduce the efficiency of the interaction of the 20-oxo group

with the catalytic site of the enzyme (decrease in k_5), as well as to reduce the binding affinity (increase in k_4/k_3).

At any rate, it is of interest that a substituent such as an oxo or a hydroxyl group at C-11 of the steroid skeleton affects the interaction with the enzyme at another, distant part of the steroid. It was shown in the previous paper that an oxo or a hydroxyl group at C-11 affects the extent of the effect of the introduction of a 17α-hydroxyl group.[1b] This may be due to the rigidity of the steroid ring.

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

The effect of a substituent at C-16 on the catalytic process, especially as regards inhibitory action on coenzyme utilization, has already been described in our previous paper.[1a] Indeed, the V_{max} value of IA-12 was very low (Table I) and, when the kinetic constants of IA-12 were compared with those of IA-1, a considerable decrease in the V_{max} value (to about 1%) was observed. The apparent K_m value also increased (23-fold) and the increase in Π value amounted to 2210-fold (Table III-a). All the other 16-substituted 20-oxo-steroids tested had no substrate activity, but had affinity for the steroid binding site.[1a] It is evident that a substituent at C-16 mainly caused a considerable decrease in the efficiency of the hydrogen transfer stage (decrease in k_5).

The role of the 17α-hydroxyl group in determining the conformational features and orientation of the 20-oxo group was also described in the previous paper.[1b] It is interesting that the effect of the introduction of a 17α-hydroxyl group on the apparent V_{max} depended on the substituent at C-21 (Table III-b).

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

	Structural change	Compd. No.	Changes in kinetic constants (fold)		
			V_{max}	K_m	Π
a)	16α-H → 16α-CH_3	IA-1 → IA-12	0.01	23	2210
b)	17α-H → 17α-OH	IA-1 → IA-2	0.42	0.92	2.2
		IVA-1 → IVA-3	0.75	0.77	1.0
		IVA-2 → IVA-4	0.73	1.1	1.5
		IA-3 → IA-4	0.54	0.36	0.66
		IIIA-6 → IIIA-7	0.82	0.14	0.17
		IB-1 → IB-2	2.6	0.67	0.26
		IB-3 → IB-4	1.8	0.33	0.18
		IB-6 → IB-7	2.9	0.55	0.19
		IIIB-3 → IIIB-4	10	1.1	0.11
c)	16-H, 17-H → Δ^{16}	IA-1 → IA-10	0.03	43	1210
d)	18-CH_3 → 18-CH_2OH	IA-1 → IA-9	0.38	228	605
		IB-1 → IB-14	0.07	65	932
e)	18-CH_3 → 18-CHO	IB-6 → IB-8	0.025	0.43	17

In the C-16/C-17 unsaturated derivative, the 17β-side chain is almost planar with respect to the steroid ring, while it is originally equatorial in the parent compound. Such a structural change resulted in a considerable decrease in the apparent V_{max} value (to one-thirtieth), an increase in the apparent K_m (43-fold), and a considerable increase in Π value (1210-fold) (Table III-c). This may be ascribable to the considerable decrease in k_5 as a result of the movement of the reacting 20-oxo group further away from the catalytic center. It seems very likely that the 20-oxo group of steroids projects towards the β-face of the steroid ring since, if it originally interacted with the enzyme at the α-face, the configuration of the 17β-side chain resulting from the introduction of a C-16/C-17 double bond would move the 20-oxo group

closer to the catalytic site of the enzyme and enhance the interaction. This finding provides further evidence that the configurational relationship between the 17β-side chain involving the 20-oxo group and the D ring (steroid ring) is very important for the efficiency of hydrogen transfer.[1a)]

Effect of Structural Changes at C-18 on the Kinetic Constants

When a methyl group at C-18 was replaced with a hydroxymethyl group, remarkable effect on the kinetic constants was observed, depending on the substituent at C-21 (Table III-d).

Among 21-deoxy derivatives, change of the substrate from IA-1 to 18-hydroxypregn-4-ene-3,20-dione (IA-9) produced a considerable increase in the apparent K_m (228-fold), while the decrease in the apparent V_{max} value was small (to two-fifths). This may be attributable to a considerable decrease in k_3 (or an increase in k_4/k_3), an increase in k_7, and a decrease in k_5. The degree of the decrease in k_5 may be slightly larger than that of the increase in k_7. Steric or polar properties of the hydroxyl group at C-18 may cause repulsion between the steroid and the binding site of the enzyme and this may also move the 20-oxo group slightly away from the catalytic center.

On the other hand, among 21-hydroxy derivatives, the substitution of a hydroxyl group for a hydrogen of C-18 [21-hydroxypregn-4-ene-3,20-dione (IB-1)→18,21-dihydroxypregn-4-ene-3,20-dione (IB-14)] resulted in a marked decrease in the apparent V_{max} (to about one-fourteenth) as well as a marked increase in the K_m value (65-fold), and the Π value increased over 930-fold. This may be ascribable to considerable decreases in k_3 (or an increase in k_4/k_3) and k_5, and possibly an increase in k_7. The degree of the decrease in k_5 may be larger than that of the increase in k_7. In molecular terms, this would mean that, in 21-hydroxy derivatives, in addition to the repulsive effect on the steroid-protein binding as seen in 21-deoxy derivatives, the hydroxyl substituent at C-18 may unfavorably affect the conformation of the 20-oxo group through interaction with the hydroxyl group at C-21. Alternatively, the hydroxyl group at C-18, in cooperation with the 21-hydroxyl group, may act to detach the reacting 20-oxo group from the catalytic center.

In any event, the steric and polar properties of the substituent at C-18 seem to play an important role in the direct interaction of the steroid with the binding site of the enzyme and also indirectly in that with the catalytic site.

An interesting feature was observed when an aldehyde group was substituted for a methyl group at C-18 of 11β,21-dihydroxypregn-4-ene-3,20-dione (IB-6) to give aldosterone (IB-8). The apparent V_{max} value of IB-6, which is relatively low, was markedly lower (one-fortieth), while the apparent K_m value, which is very high, was slightly lower (about one-half) in IB-8 (Table III-e). A slight decrease in the K_m value may be accounted for by reduction of the repulsive effect of the polar 11β-hydroxyl group on the binding interaction through the formation of an intramolecular cyclic hemiacetal resulting from the addition of a hydroxyl group at C-11 to the aldehyde carbonyl group at C-18. On the other hand, the hydroxyl group in the cyclic hemiacetal at C-18 may markedly affect both the conformational features and orientation of the 20-oxo group, probably in cooperation with the 21-hydroxyl group, as described in the case of IB-14, and may thus lead to a considerable decrease in the efficiency of hydrogen transfer (decrease in k_5).

The remarkable influence of the substituent at C-18 (axial) on the reactivity of the 20-oxo group may also be consistent with the assumption that the 20-oxo group of a steroid in the ternary complex may project towards the β-face of the steroid ring.

Assumed Interaction between 20β-Hydroxysteroid Dehydrogenase and 20-Oxo-steroid

The features of the interaction between 20β-hydroxysteroid dehydrogenase and 20-oxo-steroids, as deduced from the results of the present and previous work[1)] are shown in Fig. 1. In this scheme, pregn-4-ene-3,20-dione is shown as a representative 20-oxo-steroid fitting

the enzyme.

1) In the ternary complex, the 20-oxo group of the steroid may project towards the β-face of the steroid ring and the conformation between the 20-oxo group and the α-hydrogen of C-17 is nearly staggered, while that between the 20-methyl group (C-21) and the α-hydrogen of C-17, looking along the C-17 to C-20 axis, is in a skew form; the 20-oxo group is orientated rather far from C-18 (13β-methyl group) and more towards the β-chain of C-16. This was deduced from the finding that the substituents at the C-21 and/or at the 17α-position influenced the efficiency of the hydrogen transfer stage in various ways, depending on their contribution to the conformational features of the 20-oxo group.[1b] Changes in the kinetic constants upon structural change at C-18 (axial) and upon introduction of a C-16/C-17 double bond (Table III) provide further supporting evidence for this view.

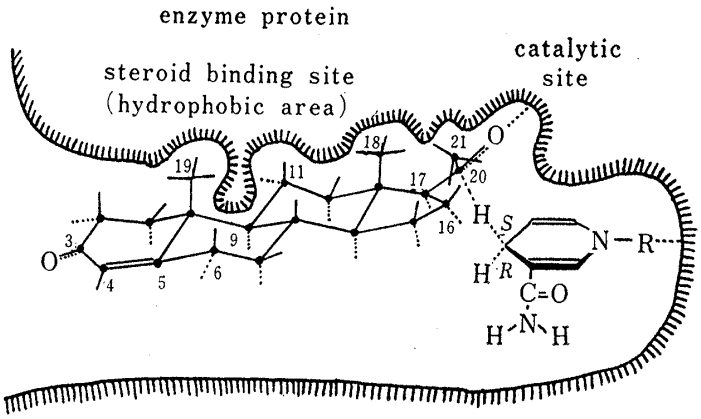

Fig. 1. Proposed Features of the Interaction between 20β-Hydroxysteroid Dehydrogenase and 20-Oxo-steroids

2) The distance between the 20-oxo group and the catalytic site may also be an important factor, since the presence of a bulky substituent at C-21 or C-17,[1a] a change in the configuration of the 17β-side chain with respect to the steroid ring (from a equatorial to a planar form), and the introduction of an 11- or an 18-hydroxyl group, may all cause movement of the 20-oxo group, directly or indirectly, from the optimum position for the catalytic reaction, resulting in the loss of, or a considerable decrease in, the efficiency of the hydrogen transfer. It is also suggested that the catalytic site of the enzyme, which interacts with C-21, C-20, and C-17, is a quite restricted region.[1a]

3) NADH on the enzyme may be located in the vicinity of C-16 of the steroid during hydrogen transfer, since the presence of any substituent at C-16, especially in the β-orientation, strongly inhibited the process of coenzyme action[1a] and, of course, the hydrogen transfer stage (Table III-a).

4) The binding site of the enzyme seems to consist of hydrophobic regions which interact with the generally hydrophobic portions of the substrate on the β-side of the B ring and in the region of C-11 (and possibly C-12). Important hydrophobic interactions may occur between the enzyme protein and methyl groups at both C-18 and C-19 of the steroid. These views are based on the findings that the polar hydroxyl group at the C-6 β-position (axial) was effective in causing a reduction of the steroid-enzyme binding[1c,2a] and the less polar oxo group at C-6 or C-7 was less effective,[2a] while the 6α-hydroxyl group (equatorial), the 6α-methyl (equatorial), the 6-methyl (planar with respect to the B ring)[1c,2a] and 9α-fluoro group[1c] were not effective. Further, the 11β-hydroxyl group (axial), the 11α-hydroxyl group (equatorial), the 11-oxo group (pseudo-equatorial), and the 11α-acetyloxyl group had a repulsive effect on the steroid-enzyme binding, decreasing in this order (Table II), and the 12α-hydroxyl group had a similar effect, as shown in Table I (IIIA-8). In addition, 19-norpregnan-20-one derivatives[1c,2a] and 18-hydroxy derivatives (Tables I and III) caused considerable decrease in the binding affinity.

5) The A ring and the B ring, except for its β-side, do not play an important role in the binding process because structural changes in these regions of substrates caused relatively small changes in the kinetic constants.[1c,2a,7]

6) The enzyme structure which interacts with the 20-oxo-steroid may be induced by the binding of NADH.[5a]

In pregnane derivatives, the steroid ring leads to overall rigidity of the molecule, while the 17β-side chain permits flexibility and conformational change for substituents at C-20 and C-21. Owing to the rigidity of the steroid ring, structural change in some part of the steroid ring may cause a change in the steroid recognition by the protein not only in this local region but also in other region(s) by altering the alignment of the steroid with the protein, as described above and previously.[1b,c] In a similar way, the configurational relationship between the steroid ring and 17β-side chain is certainly important. On the other hand, substituents at C-17α, C-18, C-20, and C-21 may affect the protein interaction not only through their chemical nature or size,[1a] but also through the conformational features and orientation of each substituent as a result of steric restrictions[1a] (Table III).

These special features of steroid structure must be an essential part of the mechanism involved in the biological action of steroids. Our work on the interaction between 20-oxosteroids and 20β-hydroxysteroid dehydrogenase may help to provide a model for a more comprehensive understanding of the molecular basis of the interactions between steroids and protein.

References and Notes

1) *a*) J. Kawamura, T. Hayakawa, and T. Tanimoto, *Chem. Pharm. Bull.*, **28**, 437 (1980); *b*) T. Hayakawa, T. Tanimoto, and J. Kawamura, *ibid.*, **28**, 730 (1980); *c*) T. Hayakawa, T. Tanimoto, T. Kimura, and J. Kawamura, *ibid.*, **29**, 456 (1981).
2) *a*) I.H. White and J. Jeffery, *Biochim. Biophys. Acta*, **296**, 604 (1973); *b*) *Idem*, *Biochem. J.*, **137**, 349 (1974).
3) G.N. Wilkinson, *Biochem. J.*, **80**, 324 (1961).
4) W.W. Cleland, *Nature* (London), **198**, 463 (1963).
5) *a*) G. Betz and J.C. Warren, *Arch. Biochem. Biophys.*, **128**, 745 (1968); *b*) G. Betz and P. Taylor, *ibid.*, **137**, 109 (1970).
6) E.L. King and C. Altman, *J. Phys. Chem.*, **60**, 1375 (1956).
7) I.H. White and J. Jeffery, *Eur. J. Biochem.*, **25**, 409 (1972).
8) T. Hayakawa, T. Tanimoto, and J. Kawamura, *Chem. Pharm. Bull.*, "in preparation".