The Altered Specificity of Cortisone Reductase with Certain *retro*Androstan-3-one Substrates

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The *retro* steroids 17 β -hydroxy-5 β ,9 β ,10 α -androstan-3-one and 5 β ,9 β ,10 α -androstan-3,17-dione were good substrates for cortisone reductase in the presence of NADH, and the products corresponded to the respective 3 β -hydroxy compounds, in which the 3 β -hydroxyl group is axial and the absolute configuration is 3*S*. The analogous natural steroids 17 β -hydroxy-5 β ,9 α ,10 β -androstan-3-one and 5 β ,9 α ,10 β -androstan-3,17-dione were very poor substrates, and gave the corresponding 3 α (equatorial, 3*R*)-hydroxy compounds, and, in the latter case, also an appreciable amount of 3 β (axial, 3*S*)-hydroxy-5 β ,9 α ,10 β -androstan-17-one. The natural steroids 17 β -hydroxy-5 α ,9 α ,10 β -androstan-3-one and 5 α ,9 α ,10 β -androstan-3,17-dione were better substrates than the *retro* steroid 17 β -hydroxy-5 α ,9 β ,10 α -androstan-3-one, but were not such good substrates as the *retro* steroids 17 β -hydroxy-5 β ,9 β ,10 α -androstan-3-one and 5 β ,9 β ,10 α -androstan-3,17-dione. Unlike these *retro* steroid 5 β ,9 β ,10 α -androstan-3-ones, the natural steroids 17 β -hydroxy-5 α ,9 α ,10 β -androstan-3-one and 5 α ,9 α ,10 β -androstan-3,17-dione gave the corresponding 3 α (axial, 3*R*)-hydroxy compounds. The *retro* steroid 17 β -hydroxy-5 α ,9 β ,10 α -androstan-3-one was not a good substrate, and the product of reaction corresponded to the 3 α (axial, 3*R*)-hydroxy compound. The nature of substrate recognition by this enzyme is discussed in the light of these structure–activity relationships.

Oxidoreductases dependent on a nicotinamide-adenine cofactor (EC 1.1.1.–) are generally acknowledged to be an important class of enzymes. The various steroid-binding proteins also constitute a broad class of proteins, in this case important because so many biological processes are subject to regulation by specific steroids. Most hydroxy steroid dehydrogenases belong to both of these classes, being oxidoreductases dependent on NAD⁺ and/or NADP⁺, and having specific steroid-binding sites, namely the substrate-binding sites. They therefore afford many points of interest, particularly concerning molecular aspects of steroid recognition and of dehydrogenase activity. The enzyme cortisone reductase (EC 1.1.1.53) shows 3 α -hydroxy steroid–NAD⁺ oxidoreductase activity towards a variety of steroids (Gibb & Jeffery, 1973*b*), and some 3 β -hydroxy steroid–NAD⁺ oxidoreductase activity in certain cases of very poor substrates (Gibb & Jeffery, 1972*c*, 1973*b*). In these steroids, as in other common saturated natural steroids, the configuration at C-10 is 10 β (10*S*), and at C-9 is 9 α (9*S* in the androstane series, but 9*R*

in the oestrane series). In the so-called *retro*androstan-3-ones, the configuration at these centres is 10 α (10*R*) and 9 β (9*R*). Because of this, the relationships between the 3-oxo group and other parts of the molecule differ in the androstane and *retro*androstan-3-one series. Nevertheless, various 3-oxo*retro*androstan-3-ones served as substrates for cortisone reductase. Their behaviour is now described, and some aspects of the substrate recognition are discussed.

Experimental

Materials

The *retro* steroids were small samples of reference materials from the collections of Philips–Duphar (Weesp, Holland) and Hoffman–La Roche (Basle, Switzerland), or were prepared (see below) from a larger quantity of *retro*testosterone (17 β -hydroxy-9 β ,10 α -androstan-4-en-3-one) kindly provided by Philips–Duphar. Crystalline preparations of cortisone reductase and nicotinamide-adenine coenzymes were obtained from Boehringer Mannheim (Mannheim, Germany). Cortisone reductase was also obtained from Sigma (London) Chemical Co. (London S.W.6,

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U.K.). Palladium-CaCO₃ and chloroiridic acid were from Johnson Matthey (London E.C.4, U.K.), NaBH₄ was from May and Baker (Dagenham, Essex, U.K.) and liquid NH₃ was from Imperial Chemical Industries (Billingham, Co. Durham, U.K.). General laboratory chemicals and solvents were the best grades available from British Drug Houses (Poole, Dorset, U.K.) or Koch-Light Laboratories (Colnbrook, Bucks., U.K.). Adsorbents for t.l.c. were from E. Merck (Darmstadt, Germany), and alumina for column chromatography was from Laporte Industries (Widnes, Lancs., U.K.).

Spectra

These were recorded with i.r. model 237 (Perkin-Elmer Ltd., Beaconsfield, Bucks., U.K.), nuclear-magnetic-resonance (n.m.r.) model HA-100 (Varian Corp., Palo Alto, Calif., U.S.A.), u.v. model SP. 800 (Pye-Unicam, Cambridge, U.K.), and circular-dichroism (c.d.) model 61 (Cary Instruments, Monrovia, Calif., U.S.A.) instruments.

Thin-layer chromatography

Layers of silica gel HF₂₅₄₊₃₆₆ and aluminium oxide G were used as previously described (Gibb & Jeffery, 1973b).

Gas-liquid chromatography

A model 104 chromatograph (Pye-Unicam) was used with a column 2m long, packed with the fluorinated silicone QF-1 (3%, w/w) on the diatomaceous earth Chromosorb W, at a temperature of 190°C, and with N₂ as carrier gas.

Enzyme reactions

These were carried out at 25°C and pH 7.0 in a sodium phosphate buffer containing dimethyl sulphoxide (10%, v/v, in preparative experiments, and 1.48% in kinetic experiments), and 150 μM-NADH, as described elsewhere (Gibb & Jeffery, 1973b). For preparation of the reaction products, 17β-hydroxy-5β,9β,10α-androstan-3-one (50 μM) was incubated with 0.037 μg of cortisone reductase/ml, 17β-hydroxy-5α,9β,10α-androstan-3-one (50 μM) with 8 μg of cortisone reductase/ml, and 5β,9β,10α-androstane-3,17-dione (50 μM) with 0.037 μg of cortisone reductase/ml. The volume of incubation medium was 250 ml in each case. The progress of reaction was assessed from the decrease in the NADH concentration, measured from the change in absorbance at 340 nm. When the rate of reaction had decreased to about zero, the NADH consumed corresponded to 85% reduction of one oxo group of the *retro* steroid

present, in each case. Products were then extracted with diethyl ether. Kinetic measurements were made over the range 30–100 μM substrate by the technique previously described (Gibb & Jeffery, 1973b), and kinetic constants were calculated by the weighting procedure of Wilkinson (1961). The range of K_m/V_{max} values allowed by 2 s.e.m. was calculated by using $(K_m + 2 \text{ s.e.m.})/(V_{max} - 2 \text{ s.e.m.})$ and $(K_m - 2 \text{ s.e.m.})/(V_{max} + 2 \text{ s.e.m.})$.

Micro-scale derivative formation

Acetylation (with acetyl chloride), oxidation (with chromic acid), and reduction (with NaBH₄) were carried out on small amounts (100–150 μg) of *retro* steroids, as previously described (Gibb & Jeffery, 1973b).

17β-Hydroxy-5β,9β,10α-androstan-3-one

*retro*Testosterone (1g) in dry ether (150 ml) was added to refluxing anhydrous liquid NH₃ (50 ml), with subsequent addition of lithium metal (0.1g) in small pieces. The mixture was stirred with exclusion of moisture until a blue colour persisted; then ethanol was added to destroy the excess of lithium. After allowing the NH₃ to evaporate, the ethereal solution was washed, and the solvent was removed. The solid residue was chromatographed on a column (25 mm diam.) of alumina (Spence grade H; Brockmann grade I/II; 100g).

After elution with benzene, and then benzene containing increasing proportions of ethyl acetate, the desired 3-ketone was eluted by benzene-ethyl acetate (23:2, v/v) and crystallized from acetone-hexane, m.p. 113–114°C [literature value for m.p. 113–114°C (Halkes & Havinga, 1965)], ν_{max} (KBr) 3360 and 1710 cm⁻¹, circular dichroism (MeOH) $\Delta\epsilon$ -1.13 (289 nm) (Found: C, 78.3; H, 10.6. Calc. for C₁₉H₃₀O₂: C, 78.6; H, 10.4%).

17β-Hydroxy-5α,9β,10α-androstan-3-one

*retro*Testosterone (1.06g) in acetonitrile (50 ml) was hydrogenated for 20 h. over a 5% Pd-CaCO₃ catalyst. T.l.c. examination of the solution showed only a trace of *retro*testosterone, and predominant formation of the desired saturated ketone. The catalyst and solvent were removed, and the crude product was purified by chromatography, essentially as for the 5β-isomer, followed by crystallization from acetone-hexane, to give 17β-hydroxy-5α,9β,10α-androstan-3-one, m.p. 123–124°C, ν_{max} (KBr) 3360 and 1073 cm⁻¹, circular dichroism (MeOH) $\Delta\epsilon$ +0.45 (290 nm) (Found: C, 78.3; H, 10.6; C₁₉H₃₀O₂ requires: C, 78.6; H, 10.4%).

Reductions with NaBH₄

General procedure. The 3-ketone (200mg) in ether (40ml), and NaBH₄ (0.25g) in methanol, were stirred together for 18h, when t.l.c. showed that reduction was complete. The product from each of the two 3-ketones was a mixture of diols (t.l.c.) in which the 3-equatorial isomer was predominant over the axial isomer by a ratio of approx 4:1. Repeated fractional crystallization from acetone-hexane and from ethyl acetate gave the 3-equatorial alcohols, although removal of the last detectable traces of axial isomers was very difficult. Samples of the diols used for reference purposes retained a few per cent of the lesser isomer in each case. Analytical samples were purified by preparative t.l.c. The products were: 5 β ,9 β ,10 α -androstan-3 α ,17 β -diol, m.p. 124–132°C after softening at 110–114°C, $\nu_{\max}$ (KBr) 3320cm⁻¹ (Found: C, 77.5; H, 10.8%, after drying at 80°C *in vacuo*; C₁₉H₃₂O₂ requires: C, 78.0; H, 11.0%); 5 α ,9 β ,10 α -androstan-3 β ,17 β -diol, m.p. 159–161°C, $\nu_{\max}$ (KBr) 3380cm⁻¹ (Found: C, 77.7; H, 10.8. C₁₉H₃₂O₂ requires: C, 78.0; H, 11.0%).

Reductions by the Henbest method

General procedure. The 3-ketone (200mg), chloro-iridic acid (0.05g), trimethyl phosphite (2ml) and aq. 90% (v/v) propan-2-ol (50ml) were heated under reflux for 62h, when t.l.c. showed almost complete disappearance of ketone. The solvents were concentrated to a small volume by evaporation under reduced pressure, and the remaining solution was diluted with water and extracted several times with ether, which gave the 3-axial alcohol contaminated with some 10% of the equatorial isomer. Fractional crystallization from acetone-hexane and preparative t.l.c. eventually yielded the desired diols, although complete removal of the equatorial isomers was again difficult. The products were: 5 β ,9 β ,10 α -androstan-3 β ,17 β -diol, m.p. 233–236°C, $\nu_{\max}$ (KBr) 3350–3310cm⁻¹ (broad) (Found: C, 77.7; H, 10.6. C₁₉H₃₂O₂ requires: C, 78.0; H, 11.0%); 5 α ,9 β ,10 α -androstan-3 α ,17 β -diol, m.p. 160–162°C, $\nu_{\max}$ (KBr) 3330cm⁻¹ (Found: C, 77.8; H, 11.2. C₁₉H₃₂O₂ requires: C, 78.0; H, 11.0%).

Results

Chemical preparation of 17 β -hydroxy-9 β ,10 α -androstan-3-ones and 9 β ,10 α -androstan-3,17 β -diols

Reduction of 17 β -hydroxy-9 β ,10 α -androstan-4-en-3-one (*retrotestosterone*) with lithium-liquid NH₃ gave 17 β -hydroxy-5 β ,9 β ,10 α -androstan-3-one. This procedure is known to give the saturated ketone with the more stable *trans*-fused ring junction (Halkes & Havinga, 1965), and the configuration at C-5 was confirmed from the negative sign of the Cotton effect

in the circular-dichroism (c.d.) curve for the ketone. Inspection of molecular models shows that the 3-ketone of the (*retro*) 5 β ,9 β ,10 α -series is enantiomeric in its A and B rings with a 3-ketone of the (natural) 5 α ,9 α ,10 β series (Jacobs & Havinga, 1972; Kirk & Klyne, 1974), which gives a positive Cotton effect of similar magnitude.

The saturated ketone of the 5 α ,9 β ,10 α -series was obtained as the main component of the mixture of isomers formed by catalytic hydrogenation of *retrotestosterone* in acetonitrile solution, over a palladium-CaCO₃ catalyst. Similar conditions favour formation of the saturated ketone with a *cis* ring junction in the (natural) 9 α ,10 β -steroid series (Combe *et al.*, 1967; Henbest *et al.*, 1967). The positive Cotton effect in the c.d. curve confirmed the 5 α -configuration, again by comparison with the quasi-enantiomeric 3-oxo 5 β ,9 α ,10 β -steroids, which show a negative Cotton effect of similar magnitude (Jacobs & Havinga, 1972; Kirk & Klyne, 1974).

Standard procedures were used to convert the two 17 β -hydroxy-9 β ,10 α -androstan-3-ones into the four required 3,17 β -diols. Reduction of unhindered cyclohexanones with NaBH₄ is known to lead to predominant formation of equatorial alcohols (Barton, 1953), in this case 5 α ,9 β ,10 α -androstan-3 β ,17 β -diol and 5 β ,9 β ,10 α -androstan-3 α ,17 β -diol from the corresponding 3-ketones. The 3-axial alcohols were obtained from the 3-ketones by the Henbest reduction procedure (chloroiridic acid-trimethyl phosphite-propan-2-ol), which is almost stereospecific in forming axial alcohols (Haddad *et al.*, 1964; Browne & Kirk, 1969). 5 α ,9 β ,10 α -Androstan-3 α ,17 β -diol and 5 β ,9 β ,10 α -androstan-3 β ,17 β -diol, obtained in this way from the corresponding 3-ketones, showed quite distinctive properties (m.p., g.l.c., t.l.c., n.m.r.) when compared with their 3-equatorial epimers.

The configurations at C-3 of the four diols were confirmed by n.m.r. examination. The spectra (determined at 100MHz in C²HCl₃) showed overlapping signals due to the methine protons at C-3 and C-17, but the addition of the shift reagent europium tris-1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedione [Eu(fod)₃] caused these signals to move to low field (Rondeau & Sievers, 1971). The C-3 proton signal was the more shifted in each case because of the greater ability of the europium complex to bind to the less-hindered 3-hydroxy group (D. N. Kirk & R. E. Morgan, unpublished work). The widths of the separated signals due to the C-3 protons then gave clear support to the assigned configurations (Bhacca & Williams, 1964); the equatorial C-3 protons in 5 α ,9 β ,10 α -androstan-3 α ,17 β -diol and 5 β ,9 β ,10 α -androstan-3 β ,17 β -diol each gave signals of width 9–10Hz, whereas the axial protons in the respective epimers at C-3 gave signals of width about 32Hz.

Because of the scarcity of material, the four 3,17-diacetates were prepared only on a micro-scale from the diols (see above) and were not fully characterized.

Nature of the enzymic reaction

The enzymic reaction product obtained from 17 β -hydroxy-5 β ,9 β ,10 α -androstan-3-one was examined by systematic t.l.c. of the compound directly, after acetylation, and after oxidation, and by g.l.c. Its behaviour was identical with that of 5 β ,9 β ,10 α -androstan-3 β ,17 β -diol and clearly distinguished it from 5 β ,9 β ,10 α -androstan-3 α ,17 β -diol. Similar examination of the product from 17 β -hydroxy-5 α ,9 β ,10 α -androstan-3-one distinguished it from 5 α ,9 β ,10 α -androstan-3 β ,17 β -diol, and showed it to behave exactly like 5 α ,9 β ,10 α -androstan-3 α ,17 β -diol. In neither of these cases was any other product detected. It is concluded that the enzymic reaction with 17 β -hydroxy-5 β ,9 β ,10 α -androstan-3-one gave the corresponding 3 β (axial, 3*S*)-hydroxy compound, whereas reaction of 17 β -hydroxy-5 α ,9 β ,10 α -androstan-3-one gave the corresponding 3 α (axial, 3*R*)-hydroxy compound. This contrasted with reaction of 17 β -hydroxy-5 β ,9 α ,10 β -androstan-3-one, which gave the corresponding 3 α (equatorial, 3*R*)-hydroxy compound; 17 β -hydroxy-5 α ,9 α ,10 β -androstan-3-one gave the corresponding 3 α (axial, 3*R*)-hydroxy compound (Gibb & Jeffery, 1973*b*).

Characterization of the enzymic-reaction product obtained from 5 β ,9 β ,10 α -androstan-3,17-dione was complicated by lack of reference samples of the 3-hydroxy-5 β ,9 β ,10 α -androstan-17-ones and 17 α -hydroxy-5 β ,9 β ,10 α -androstan-3-one. The enzymic-reaction product was therefore examined by systematic t.l.c. directly, after reduction, after acetylation, and after reduction followed by acetylation. This showed that it was not 17 β -hydroxy-5 β ,9 β ,10 α -androstan-3-one, that it appeared to have one acetylatable hydroxyl group (from the mobility of the acetylation product), and that it was probably a 17-ketone [from the typical violet colour of its Zimmermann (1935) reaction]. This suggested that

it was one of the two 3-hydroxy-5 β ,9 β ,10 α -androstan-17-ones. The micro-scale borohydride reduction of the enzymic-reaction product gave material that was distinguished from 5 β ,9 β ,10 α -androstan-3 α ,17 β -diol, and behaved exactly like 5 β ,9 β ,10 α -androstan-3 β ,17 β -diol. This would be consistent with the view that the enzymic-reaction product was 3 β -hydroxy-5 β ,9 β ,10 α -androstan-17-one. The borohydride reduction of the most likely alternative compound, namely 3 α -hydroxy-5 β ,9 β ,10 α -androstan-17-one, would be expected to give 5 β ,9 β ,10 α -androstan-3 α ,17 β -diol. Thus, although in this case the identification of the enzymic-reaction product was not wholly unequivocal, it strongly suggested that the enzymic reduction of 5 β ,9 β ,10 α -androstan-3,17-dione followed a course analogous to the enzymic reduction of 17 β -hydroxy-5 β ,9 β ,10 α -androstan-3-one and gave the corresponding 3 β (axial, 3*S*)-hydroxy compound. The enzymic reduction of 5 β ,9 α ,10 β -androstan-3,17-dione gave a mixture of the corresponding 3 α (equatorial, 3*R*)-hydroxy and 3 β (axial, 3*S*) compounds (Gibb & Jeffery, 1973*b*).

Rate of the reaction

The two 5 β ,9 β ,10 α -androstan-3-ones were good substrates (Table 1), being utilized some 140 times more efficiently than the corresponding 5 β ,9 α ,10 β -androstan-3-ones (Gibb & Jeffery, 1973*b*). They were even utilized some 5–26 times more efficiently than the corresponding 5 α ,9 α ,10 β -androstan-3-ones, the difference here arising from the lower $V_{\max}$ values of the latter (Gibb & Jeffery, 1973*b*). (A lower value of $K_m/V_{\max}$ corresponds to more efficient substrate utilization.)

The third *retro* steroid substrate, 17 β -hydroxy-5 α ,9 β ,10 α -androstan-3-one, was utilized some 60 times less efficiently than the other two *retro* steroids. This was because of both its higher K_m and its lower $V_{\max}$ values (Table 1). It was a poorer substrate than 17 β -hydroxy-5 α ,9 α ,10 β -androstan-3-one also because of its higher K_m and lower $V_{\max}$ values (Gibb & Jeffery, 1973*b*), but it was utilized a little more

Table 1. *Kinetic constants for the reduction of various retroandrostan-3-ones by cortisone reductase*

Conditions were: 25°C, pH 7.0, 100mM-sodium phosphate buffer, 1.48% (v/v) dimethyl sulphoxide, 150 μ M-NADH, 30–100 μ M substrate.

Substrate	Correlation coefficient of reciprocal rate against reciprocal substrate concentration	$K_m \pm 2 \text{ s.e.m.}$ (μ M)	$V_{\max} \pm 2 \text{ s.e.m.}$ ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	$K_m/V_{\max}$ (range allowed by $\pm 2 \text{ s.e.m.}$ in K_m and $V_{\max}$)
17 β -Hydroxy-5 β ,9 β ,10 α -androstan-3-one	0.99	124 $\pm$ 18.6	76.3 $\pm$ 8.2	1.62 (1.25–2.1)
5 β ,9 β ,10 α -Androstane-3,17-dione	0.91	101 $\pm$ 56	54.8 $\pm$ 19	1.84 (0.75–4.4)
17 β -Hydroxy-5 α ,9 β ,10 α -androstan-3-one	0.99	439 $\pm$ 196	4.31 $\pm$ 1.65	102 (44–240)

efficiently than 17β -hydroxy- $5\beta,9\alpha,10\beta$ -androstan-3-one, in this case its higher $V_{\max}$ value outweighing the higher K_m value. Thus 17β -hydroxy- $5\alpha,9\beta,10\alpha$ -androstan-3-one was a poor substrate, but not exceptionally poor, whereas 17β -hydroxy- $5\beta,9\beta,10\alpha$ -androstan-3-one and $5\beta,9\beta,10\alpha$ -androstan-3,17-dione were among the best substrates known to undergo reduction at the 3-position by this enzyme.

Site of the reaction

Cortisone reductase has 20β -hydroxy steroid-NAD⁺ oxidoreductase activity (White & Jeffery, 1973) as well as 3-hydroxy steroid-NAD⁺ oxidoreductase activities (Gibb & Jeffery, 1972c, 1973b). Kinetic findings showed the various activities to be closely related, and possibly to depend on the same

Fig. 1. Superposed models of 17β -hydroxy- $5\alpha,9\alpha,10\beta$ -androstan-3-one (I) and 17β -hydroxy- $5\alpha,9\beta,10\alpha$ -androstan-3-one (II)

The numbering is that customary for steroids, the unprimed numbers relating to compound (I) and the primed numbers to compound (II). The bonds are shown common to both molecules (—), those found only in compound (I) (----), or those found only in compound (II) (····).

Fig. 2. Superposed models of 17β -hydroxy- $5\alpha,9\alpha,10\beta$ -androstan-3-one (I) and 17β -hydroxy- $5\beta,9\beta,10\alpha$ -androstan-3-one (III)

The numbering is that customary for steroids, the unprimed numbers relating to compound (I) and the primed numbers to compound (III). The bonds are shown common to both molecules (—), those found only in compound (I) (----), or those found only in compound (III) (····).

active centre (Gibb & Jeffery, 1971, 1972a), though it was not possible to establish this for the very limited 3β -hydroxy steroid-NAD⁺ oxidoreductase activity. The effect of $15\ \mu\text{M}$ - 20β -hydroxypregn-4-en-3-one (the product of the enzymic reaction with pregn-4-ene-3,20-dione) on the initial rate of reduction of $30\ \mu\text{M}$ - 17β -hydroxy- $5\beta,9\beta,10\alpha$ -androstan-3-one was therefore determined; the rate was decreased from 22.5 to $5.1\ \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. It is therefore unlikely that this 3β (axial, $3S$)-hydroxy steroid-NAD⁺ oxidoreductase activity of cortisone reductase involves an active centre independent of the active centre for the 20β -hydroxy steroid-NAD⁺ oxidoreductase activity, and it is likely that the 3α -, 3β - and 20β -hydroxy steroid-NAD⁺ oxidoreductase activities and the adamantan-2-ol-NAD⁺ oxidoreductase activity of cortisone reductase all involve either the same active centre or centres which, if they are topographically distinct, nevertheless do not function independently.

Discussion

Examination of molecular models based on essentially undistorted all-chair conformations shows that the A rings of 17β -hydroxy- $5\alpha,9\beta,10\alpha$ -androstan-3-one (*retro* series) and 17β -hydroxy- $5\alpha,9\alpha,10\beta$ -androstan-3-one (natural series) can be exactly superposed by placing them both β -face upwards, so that C-1, C-2, C-3, C-4, C-5 and C-6 in the two molecules coincide, and the other parts of the molecules then diverge (Fig. 1). Thus C-9 of the former occupies about the same position as C-19 of the latter, and C-17 of the former comes close to C-18 of the latter. In order that the enzyme may transfer hydrogen to form, in both these cases, the 3α -hydroxy compound, it seems likely that the A rings become located in a similar way in the catalytic region of the enzyme. From models this can be seen to require that the region of the enzyme in which the C rings become located should either provide a binding site some $0.6\ \text{nm}$ in size (α -face to β -face) or should be flexible. The decrease in V_{max} for the series 17β -hydroxy- $5\alpha,9\alpha,10\beta$ -oestrane-3-one, 17β -hydroxy- $5\alpha,9\alpha,10\beta$ -androstan-3-one and 17β -hydroxy- $5\alpha,9\beta,10\alpha$ -androstan-3-one ($134, 14, 4.3\ \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) (Gibb & Jeffery, 1973b; and Table 1) could be explained by supposing that the C-19 group of 17β -hydroxy- $5\alpha,9\alpha,10\beta$ -androstan-3-one and the similarly located C- and D-ring portion of 17β -hydroxy- $5\alpha,9\beta,10\alpha$ -androstan-3-one (Fig. 1) caused changes in the exact positioning of the A ring in the catalytic region, decreasing the ease of hydrogen transfer.

The A- and B-ring portion of 17β -hydroxy- $5\beta,9\beta,10\alpha$ -androstan-3-one is the mirror image of the A- and B-ring portion of 17β -hydroxy- $5\alpha,9\alpha,10\beta$ -androstan-3-one. The A- and B-ring portions of the enzymic reaction products obtained from these substrates,

namely $5\beta,9\beta,10\alpha$ -androstan- $3\beta,17\beta$ -diol and $5\alpha,9\alpha,10\beta$ -androstan- $3\alpha,17\beta$ -diol, are also mirror images. The formal simplicity of this relationship is pleasing, but in itself perhaps reveals little about the molecular course of events. The 4-pro-*S* hydrogen of the NADH is transferred in all cases (Gibb & Jeffery, 1973a).

Inspection of models shows that the A rings of the $5\beta,9\beta,10\alpha$ -androstan- and $5\alpha,9\alpha,10\beta$ -androstan- compounds can be exactly superposed by placing the former with its β -face down, and the latter with its β -face up, so that C-5, C-4, C-3, C-2 and C-1 of the $5\beta,9\beta,10\alpha$ compound coincide with C-1, C-2, C-3, C-4 and C-5 respectively, of the $5\alpha,9\alpha,10\beta$ -compound (Fig. 2). The C-19 groups of the compounds then coincide, but the other parts of the molecules diverge (although the C-18 carbon atoms are similarly located). If this were the way in which these steroids became bound at the active centre of the enzyme, then hydrogen transfer in an exactly similar manner would convert a $5\beta,9\beta,10\alpha$ -androstan-3-one into the corresponding $5\beta,9\beta,10\alpha$ -androstan- 3β -ol, and a $5\alpha,9\alpha,10\beta$ -androstan-3-one into the corresponding $5\alpha,9\alpha,10\beta$ -androstan- 3α -ol. These were the products found. However, two points may be made against such an explanation. First, the fact that 17β -hydroxy- $5\alpha,9\alpha,10\beta$ -oestrane-3-one was a better substrate than 17β -hydroxy- $5\alpha,9\alpha,10\beta$ -androstan-3-one (Gibb & Jeffery, 1973b) might lead, following the structural alignment suggested above (Fig. 2), to the expectation that 17β -hydroxy- $5\beta,9\beta,10\alpha$ -androstan-3-one would be a worse substrate than 17β -hydroxy- $5\alpha,9\alpha,10\beta$ -androstan-3-one. In fact, it was better. Second, C-6 of 17β -hydroxy- $5\beta,9\beta,10\alpha$ -androstan-3-one occupies, in this arrangement, about the position that the 1β -methyl group of 17β -hydroxy- 1β -methyl- $5\alpha,9\alpha,10\beta$ -androstan-3-one would be expected to occupy. Thus the latter would be expected to be quite a good substrate, whereas it was not (Gibb & Jeffery, 1972b). If, however, the binding site were flexible so that the fit of the A ring at the catalytic region were influenced by conformational changes induced in the enzyme by parts of the substrate quite remote from ring A, then this apparent conflict might be resolved. The concept of a protein having a flexible and generally hydrophobic region is not out of keeping with current ideas of enzyme structure.

The almost-rigid nature of these steroid substrates suggests the suitability of this enzyme for studying the phenomenon of induced fit. The possible value of such studies as a model to aid the understanding of the less-accessible non-enzymic steroid-receptor proteins involved in regulatory mechanisms is also apparent.

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