Structural Features of Ring C of 20-Oxo Steroids and the Interaction with Cortisone Reductase

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Kinetic measurements were made with cortisone reductase (20-dihydrocortisone–NAD+ oxidoreductase, EC 1.1.1.53) and a series of substrates which differed in shape, size and electronic character in the region adjacent to C-11, C-14 and C-18. Structural changes at C-11 in these substrates resulted in up to 660-fold changes in the apparent \( K_m \) value, up to 200-fold changes in the apparent \( V_{max} \) value and up to 800-fold changes in the ratio of these kinetic constants. It is suggested that interactions important for substrate function normally occur between the enzyme and the C ring in the region of C-11, that these interactions arise from 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 contains a polar substituent in this portion of the molecule, then polar interactions with polar moieties of the enzyme can also be important. It is further suggested that the part of the enzyme that interacts with the region of C-11 in the substrate is flexible, and that substrate binding involves at least some degree of induced fit.

Experimental

Enzymic reactions

Initial-rate determinations. These were made as described by White & Jeffery (1973a).

Determination of apparent \( K_m \) and apparent \( V_{max} \) values. The initial reaction rate was determined in duplicate at six substrate concentrations covering as wide a range as possible, and each such experiment was repeated at least once so that the pooled results gave at least 24 points for each substrate. Linear regressions of reciprocal initial reaction rate against reciprocal substrate concentration were calculated without weighting, or by using the weighting procedure of Wilkinson (1961). The apparent \( V_{max} \) and minus the apparent \( K_m \) values were the reciprocals of the intercepts of these regression lines with the ordinate and abscissa axes respectively (cf. Lineweaver & Burk, 1934). Standard errors of the mean of apparent \( V_{max} \) and apparent \( K_m \) values were estimated by Wilkinson's (1961) method. However, when \( K_m \) is high compared with the substrate concentrations used, this method gives high values for the standard errors, and 2 S.E.M. may approach or even exceed the mean value of \( K_m \) and \( V_{max} \). Such estimates are misleading, the reality being that \( K_m \) and \( V_{max} \) may lie somewhat above the estimated mean values in such cases but cannot be very low or negative. The following procedure was
therefore devised, and was used for 11α-(3-carboxypropanoyloxy)pregn-4-ene-3,20-dione. The initial rate of reaction, \( v \), is related to the substrate concentration, \( s \), by the equation:

\[
1/v = \Pi 1/s + 1/V_{\text{max}}.
\]  

(1)

where \( \Pi \) is the ratio of the apparent \( K_m \) and apparent \( V_{\text{max}} \)

values. It follows that from any two values (\( s_1, v_1 \) and \( s_2, v_2 \)) an estimate of \( \Pi \) may be obtained:

\[
\Pi = (1/v_1 - 1/v_2)/(1/s_1 - 1/s_2)
\]  

(2)

The four values of \( v \) obtained experimentally for each of the six values of \( s \) were averaged to give six mean values \( \bar{v} \). From these, all the 15 different pairs (that is 6 \( C_5 \)) were used in eqn. (2) to give 15 estimates of \( \Pi \), which were then averaged to give \( \bar{\Pi} \). Six estimates of \( V_{\text{max}} \) were then obtained by using this value of \( \bar{\Pi} \), the six values of \( \bar{v} \), and the following equation, based on eqn. (1):

\[
1/V_{\text{max}} = 1/\bar{v} - \bar{\Pi} (1/s)
\]

From these six values of \( V_{\text{max}} \), six values of \( K_m \) were obtained by using the equation:

\[
K_m = \bar{\Pi} \cdot V_{\text{max}}.
\]

The six values of \( V_{\text{max}} \) and \( K_m \) were then averaged to give mean values \( \bar{V}_{\text{max}} \) and \( \bar{K}_m \) and standard errors were calculated by using the formulae:

\[
\text{S.E.M. of } V_{\text{max}} = \frac{1}{6} \left[ \Sigma (V_{\text{max}} - \bar{V}_{\text{max}})^2 \right]^{1/2}
\]

\[
\text{S.E.M. of } K_m = \frac{1}{6} \left[ \Sigma (K_m - \bar{K}_m)^2 \right]^{1/2}
\]

The maximum range of \( \Pi \) allowed by 2 s.e.m. variation in \( K_m \) and \( V_{\text{max}} \) values was then calculated by using \((\bar{K}_m + 2 \times \text{s.e.m.})/(\bar{V}_{\text{max}} - 2 \times \text{s.e.m.})\) and \((\bar{K}_m - 2 \times \text{s.e.m.})/(\bar{V}_{\text{max}} + 2 \times \text{s.e.m.})\), to obtain the relatively broad estimate of the range of \( \Pi \) used in other cases.

Preparation of enzymic reaction products. The reaction was carried out essentially as in the kinetic experiments (White & Jeffery, 1973a) but at pH 5.4, in 20% (v/v) dimethyl sulphoxide, and on a larger scale (up to 54ml of reaction mixture). The progress of the reaction was followed as in the kinetic experiments, but with portions (2.7ml) from the reaction mixture. When the rate of reaction had decreased to about zero (usually after about 0.5h), the reaction mixture was extracted with diethyl ether (3 × 10ml), the extract washed with water (2 × 0.5ml), dried over Na\(_2\)SO\(_4\), and the solvent removed by rotary evaporation. The residue was dissolved in methanol and portions were examined by t.l.c., the u.v. spectrum was measured, and derivatives were made on a micro scale and examined by t.l.c.

Characterization of enzymic reaction products

Ultraviolet spectra. These were recorded with a model SP. 800 spectrophotometer (Pye–Unicam Ltd., Cambridge, Cambs., U.K.).

Melting points. These were determined by using a Koefler block.

Thin-layer chromatography. T.l.c. was carried out by ascending development for 15 cm in a closed tank at 23°C. In some cases, chromatograms were developed twice or three times in the same direction.

Micro-scale formation of derivatives of steroids. This was carried out as follows. (1) Hydrolysis. The steroid (up to 0.5mg) was dissolved in 4% (w/v) NaOH in ethanol (0.50ml) and left for 15h at 23°C. Acetic acid (30 \( \mu \)l) and diethyl ether (5ml) were then added, and the mixture was washed with water (5ml), dried over Na\(_2\)SO\(_4\), and the solvent removed by rotary evaporation. (2) Acetylation. The steroid (up to 0.5mg) was dissolved in dry pyridine (0.4ml) and acetic anhydride (0.2ml) added. The solution was left for 15h at 23°C, then warmed to about 50°C and evaporated to dryness in a stream of N\(_2\). (3) Oxidation. This was as described by Gibb & Jeffery (1973b).

Results

As judged by the disappearance of NADH, 14α-hydroxypregn-4-ene-3,20-dione and 18-hydroxy-pregn-4-ene-3,20-dione were reduced. The former steroid came out of solution at 10 \( \mu \)M. The 18-hydroxy-pregn-4-ene-3,20-dione exists in three tautomeric forms, so the reacting form was uncertain. Kinetic constants were not obtained for these two steroids, and the reaction products were not investigated.

In all of the other cases, the reaction of the 20-oxo steroids consumed an amount of NADH corresponding to more than 80% but not more than 100% reduction of one oxo group, the values found being as follows: pregn-4-ene-3,20-dione (84%); pregn-4-ene-11,20-trione (97%); 11α-acetoxypregn-4-ene-3,20-dione (97%); 11α-hydroxy pregn-4-ene-3,20-dione (95%); 11β-hydroxy pregn-4-ene-3,20-dione (95%); 11α-(3-carboxypropanoyloxy)pregn-4-ene-3,20-dione (92%); 9,11-secopregn-4-ene-3,20-dione (92%). The u.v.-absorption maximum close to 240nm indicated that the 4-en-3-one moiety was present in the reaction product in every case. The behaviour of the products and their derivatives on t.l.c. was consistent with the view that each product was a 20-hydroxy compound corresponding to the 20-oxo substrate. In particular, the product from pregn-4-ene-3,11,20-trione was not 11α- or 11β-hydroxy pregn-4-ene-3,20-dione; the products from 11α-acetoxy pregn-4-ene-3,20-dione and 11α-(3-carboxypropanoyloxy)pregn-4-ene-3,20-dione were different from the product from 11α-hydroxy pregn-4-ene-3,20-dione, but after hydrolysis they were not
distinguished from the latter. No reaction was detected with 11α-hydroxypregn-4-ene-3,20-dione or 11β-hydroxypregn-4-ene-3,20-dione as test substrate in the presence of NAD⁺ (2 mM) and with 500 times the amount of enzyme with which pregn-4-ene-3,11,20-trione reacted rapidly in the presence of NADH (150 μM). The reactions therefore appeared to be reduction of the 20-oxo group only. With pregn-4-ene-3,20-dione it was rigorously established that the product was 20β-hydroxypregn-4-ene-3,11,20-trione (White & Jeffery, 1973b), and for two related 20-oxo steroid substrates the products were shown not to be the 20α-hydroxy compounds, whether as test substrates or with 3,11,20-trione (30–100 μM). NADH reacted rapidly with 3,11,20-trione (Jeffery, 1973b), and for two 20α-hydroxy compounds, whether as test substrates or with 20α-hydroxy pregn-4-ene-3,20-dione as substrate (10 μM), and 2.0 ± 0.2 μM (± 2 S.E.M.) with pregn-4-ene-3,20-dione as substrate (45 μM). The corresponding apparent Vₘₐₓ values were 7.3 ± 0.2 and 6.0 ± 0.2 μmol·min⁻¹·mg⁻¹.

Discussion

Interpretation of the kinetic constants

The reaction probably follows mainly an ordered course in which the enzyme first binds the coenzyme and then the steroid, hydrogen is transferred, and the

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**Table 1. Kinetic constants for reduction of the 20-oxo group of various steroids by cortisone reductase**

The principal reaction conditions were: pH 7.0; 25°C; 0.1 M-phosphate buffer; 10% (v/v) dimethyl sulphoxide; 150 μM-NADH; 20-oxo steroid as shown in the table. The kinetic constants shown with S.E.M. in the table were calculated by using the method of Wilkinson (1961), except for those denoted by *, for which a new method (described in the text) was used.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Apparent Vₘₐₓ (μmol·min⁻¹·mg of enzyme⁻¹)</th>
<th>Apparent Kₘ (μM)</th>
<th>Π (with maximum range allowed by 2S.E.M. change in Kₘ and Vₘₐₓ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-weighted</td>
<td>Weighted ± 2 S.E.M.</td>
<td>Non-weighted</td>
</tr>
<tr>
<td>Preg-4-ene-3,20-dione (1–45 μM)</td>
<td>6.2</td>
<td>6.1 ± 0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Preg-4-ene-3,11,20-trione (30–100 μM)</td>
<td>66.8</td>
<td>66.0 ± 4.3</td>
<td>52.3</td>
</tr>
<tr>
<td>11α-Acetoxy pregn-4-ene-3,20-dione (10–100 μM)</td>
<td>8.6</td>
<td>8.6 ± 0.3</td>
<td>20.3</td>
</tr>
<tr>
<td>11α-Hydroxy pregn-4-ene-3,20-dione (30–100 μM)</td>
<td>11.0</td>
<td>11.6 ± 0.9</td>
<td>145</td>
</tr>
<tr>
<td>11β-Hydroxy pregn-4-ene-3,20-dione (17–60 μM)</td>
<td>8.3</td>
<td>7.8 ± 1.1</td>
<td>187</td>
</tr>
<tr>
<td>11α(3-Carboxypropyloxy) pregn-4-ene-3,20-dione (15–60 μM)</td>
<td>8.6</td>
<td>8.5 ± 6.4</td>
<td>702</td>
</tr>
<tr>
<td>9,11-Secopregn-4-ene-3,20-dione (20–80 μM)</td>
<td>0.34</td>
<td>0.33 ± 0.02</td>
<td>50.6</td>
</tr>
</tbody>
</table>

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steroid product leaves the enzyme followed by the coenzyme product (Betz & Warren, 1968; Betz & Taylor, 1970). This is represented in Scheme 1, in which E is enzyme, B is 20-oxo steroid, P is 20β-hydroxy steroid, Q is NAD⁺, and A is NADH (although the reaction involves NADH + H⁺, it is generally agreed that acquisition of the proton need not be represented as a separate step). It is assumed that the mechanism of reaction is essentially the same for all the substrates of the present study.

Application of steady-state methods (King & Altman, 1956) to this reaction mechanism gives the apparent \( K_m \) and apparent \( V_{\text{max}} \) values in terms of the individual rate constants as follows.

Apparent \( K_m \) value for B:

\[
k_{+5}(k_{-2}k_{-3} + k_{-2}k_{+4} + k_{+3}k_{+6})(k_{-1} + k_{+1}[A])
\]

\[
k_{+2}(k_{+3}k_{+4}k_{+5} + k_{+1}(k_{+3}k_{+4} + k_{+3}k_{+5} + k_{-3}k_{+5} + k_{+4}k_{+5})[A])
\]

Apparent \( K_m \) value for A:

\[
k_{+2}k_{+3}k_{+4}k_{+5}[B] + k_{-1}k_{+5}(k_{-2}k_{-3} + k_{-2}k_{+4} + k_{+3}k_{+4})
\]

\[
k_{+1}(k_{+3}k_{+4} + k_{+3}k_{+5} + k_{-3}k_{+5} + k_{+4}k_{+5})[B] + k_{+1}k_{+5}(k_{-2}k_{-3} + k_{-2}k_{+4} + k_{+3}k_{+4})
\]

Apparent \( V_{\text{max}} \) value for B:

\[
\frac{[E_{\text{total}}] \times k_{+1}k_{+3}k_{+4}k_{+5}[A]}{k_{+1}(k_{+3}k_{+4} + k_{+3}k_{+5} + k_{-3}k_{+5} + k_{+4}k_{+5})[A] + k_{+3}k_{+4}k_{+5}}
\]

(5)

The ratio of apparent \( K_m \) for B to apparent \( V_{\text{max}} \) for B at the same concentration of A is conveniently abbreviated as \( \Pi \):

\[
\Pi = \frac{(k_{-2}k_{-3} + k_{-2}k_{+4} + k_{+3}k_{+4})(k_{-1} + k_{+1}[A])}{[E_{\text{total}}] \times k_{+1}k_{+2}k_{+3}k_{+4}[A]}
\]

(6)

In the present experiments, A (NADH) was used at the fixed concentration of 150 \( \mu M \) when the concentration of B (20-oxo steroid) was varied. The apparent \( K_m \) value for A found in the present study (see the Results section) and previous studies (White & Jeffery, 1972, 1973a) was in the region of 2.3 \( \mu M \) for various 20-oxo steroid substrates. The approximation that 150 \( \mu M \) was a saturating concentration of A therefore seems justified, and eqns. (3) and (5) can be simplified as follows.

Apparent \( K_m \) value for B:

\[
\frac{(k_{-2}k_{-3} + k_{-2}k_{+4} + k_{+3}k_{+4})k_{+5}}{(k_{+3}k_{+4} + k_{+3}k_{+5} + k_{-3}k_{+5} + k_{+4}k_{+5})k_{+2}}
\]

(7)

Apparent \( V_{\text{max}} \) value for B:

\[
\frac{[E_{\text{total}}] \times k_{+3}k_{+4}k_{+5}}{(k_{+3}k_{+4} + k_{+3}k_{+5} + k_{-3}k_{+5} + k_{+4}k_{+5})}
\]

(8)

\[
\Pi = \frac{(k_{-2}k_{-3} + k_{-2}k_{+4} + k_{+3}k_{+4})}{[E_{\text{total}}] \times k_{+3}k_{+4}k_{+5}}
\]

(9)

Scheme 1. The principal course of reaction

E is cortisone reductase, B 20-oxo steroid, P 20β-hydroxy steroid, Q NAD⁺ and A NADH (with which H⁺ is also required).

From eqns. (7) and (8) it can be seen that \( k_{+4} \), which is a constant concerned with binding of steroid to the enzyme, occurs in both the expression for the apparent \( K_m \) and that for the apparent \( V_{\text{max}} \), values, and in each case it occurs both in the numerator and in the denominator. It is clear that, in general, the effect upon the apparent \( K_m \) and apparent \( V_{\text{max}} \) of a change in \( k_{+4} \) is not easily predicted. In some cases it may seem likely that a change of steroid structure that alters \( k_{+4} \) may also alter \( k_{-2} \) in a similar way. However, this need not be so; \( k_{+4} \) and \( k_{-2} \) do not relate to the same enzyme complex. One conclusion to be drawn from such considerations is that it is pointless to seek to interpret precisely any small changes in \( K_m \) or \( V_{\text{max}} \) values. This has therefore not been attempted. When substantial changes occur, these must be one of eight types, namely: \( K_m \) and \( V_{\text{max}} \) both up, or both down, or \( K_m \) up and \( V_{\text{max}} \) down, or \( K_m \) down and \( V_{\text{max}} \) up, or \( K_m \) up and \( V_{\text{max}} \) little changed, or \( K_m \) down and \( V_{\text{max}} \) little changed, or \( K_m \) little changed and \( V_{\text{max}} \) up, or \( K_m \) little changed and \( V_{\text{max}} \) down. These changes can then be considered in relation to eqns. (7) and (8).

Structure–activity relationships

Comparison of the kinetic constants (Table 1) for pregn-4-ene-3,20-dione, 11α-hydroxypregn-4-ene-3,20-dione and 11β-hydroxyprogren-4-ene-3,20-dione shows that all three compounds had roughly similar apparent \( V_{\text{max}} \) values (within a factor of 2), and that the apparent \( K_m \) values of the two 11-hydroxy compounds are similar to each other, but differ considerably from that of pregn-4-ene-3,20-dione (factors of more than 100). Inspection of eqns. (7)–(9) shows that this could arise if the 11-hydroxyl groups caused an increase in \( k_{-2}/k_{+2} \).
and in $k_{+4}$. In molecular terms, this would mean that the 11-hydroxy steroids did not bind to the enzyme as well as the parent (11-deoxy) compound. The position (relative to the rest of the steroid) of the equatorial 11α-hydroxy group and the sterically hindered axial 11β-hydroxy groups differ considerably. The similarity of their effects on the apparent $K_m$ and apparent $V_{max}$ values suggests that the effect does not involve any precisely oriented interaction with the hydroxyl group. The effect of the 11-hydroxy groups was much greater than the effect of 6-hydroxyl groups (White & Jeffery, 1973a), which argues against a wholly non-specific polar-group effect. Indeed, the greater effect of the sterically-hindered axial 6β- and 11β-hydroxy groups than of the corresponding equatorial 6α- and 11α-hydroxy groups, respectively, would be consistent with a hydrophobic type of interaction involving the β-side of rings B and C in the unsubstituted compound. Consistent with this, and allowing the concept to be extended, the 11α-acetoxyl compound had a lower apparent $K_m$ value than the 11α-hydroxyl compound, and a roughly similar apparent $V_{max}$ value, so that one could think of the bulkier, still-polar, but less-hydrophilic acetoxyl group as being less deleterious to binding in a hydrophobic pocket than was an 11α-hydroxyl group, though less favourable for binding than the absence of substituents. The 11α-(3-carboxypropanoyloxy) compound had a much higher apparent $K_m$ value than the 11α-hydroxyl compound, but again the apparent $V_{max}$ value was roughly similar. In this case, the substituent was attached at the 11α-position by the polar ester linkage, and bore a carboxyl group, the position of which relative to the rest of the steroid could not be specified because of the flexible connecting carbon chain, some 0.5 nm in length. The overall effect could not therefore be apportioned with any confidence between the effect of the carboxyl group itself, the effect of its actual location (allowed by the flexible chain) and the effect of the bulk of the whole substituent group. The fact that this 11α-(3-carboxypropanoyloxy) compound was a substrate established that the enzyme could tolerate a large and highly polar group extending from the 11α-position. Comparison with the 11-oxo compound is now valuable. In this case, the substituent group was smaller than a hydroxyl group, and a little less hydrophilic, though these differences are minor compared with some of those noted above. The 11-oxo group is located (pseudoequatorially) between the positions that an 11α-hydroxyl and 11β-hydroxyl group would occupy. The apparent $K_m$ value was much lower than for either of the 11-hydroxyl compounds, and, lying between the apparent $K_m$ values of the 11α-acetoxyl and 11α-hydroxy compounds, could perhaps be accounted for in terms of general hydrophilic character. However, the apparent $V_{max}$ value of the 11-oxo compound was the highest of the series, being more than fivefold greater than the next highest (the 11α-hydroxy compound). It is extremely difficult to imagine how the small and polar 11-oxo group could exert such a striking effect unless it lay close enough to some part of the enzyme to participate in a highly specific interaction of some kind. It is clear from the substrate behaviour of the 11α-acetoxyl and 11α-(3-carboxypropanoyloxy) compounds that close approach of the enzyme to the α face near C-11 is not essential for substrate function. These findings and interpretations are not in conflict if it is supposed that a flexible part of the enzyme lies in the region of C-11, so that the substrate itself determines the shape of this part of its binding site.

The 9,11-seco compound contains the A, B and D ring structure identical with that of the other substrates studied. Unlike the other compounds, however, the A and B ring portion can rotate (about the C-8--C-14 bond) altering its position relative to the D ring and the reacting 20-oxo group. When bound to the enzyme, this molecule might have a shape somewhat similar to or considerably different from that of the intact steroids, but the shape could not be exactly similar to that of the intact steroids because of the extra hydrogen atoms at C-11 and C-9β. It is therefore noteworthy that whereas pregn-4ene-3,20-dione was the best substrate of the series (lowest $V_{max}$ value), 9,11-secopregn-4-ene-3,20-dione was the worst (highest $V_{max}$ value). The lower apparent $V_{max}$ (about 20-fold) and the higher apparent $K_m$ (about 40-fold) could arise from an increase in $k_{-3}/k_{+3}$ and $k_{-2}/k_{+2}$. This would mean that the 9,11-seco steroid did not bind to the enzyme as well as the parent steroid, and that hydrogen was not transferred as efficiently to the bound seco steroid. Nevertheless, the 9,11-seco steroid probably bound better than the 11-hydroxy compounds. The view that an essentially hydrophobic part of the enzyme lies in the region of the C ring of the bound steroid would be consistent with this. The 9,11-seco steroid, because of its less rigid structure, could fail to induce in the enzyme a conformational change as favourable for reaction as the changes induced by the intact steroids. The finding that the 14α-hydroxy and 18-hydroxy compounds were substrates extend the finding for the 11α-hydroxy and 11β-hydroxy compounds, showing that a polar group on either side of the C ring did not prevent reaction. The 18-hydroxy compound was particularly interesting because of the relationship of the 18-hydroxyl group to the 20-oxo group.

Interactions between the enzyme and the region of the C ring of the substrate can certainly have a marked influence on the enzymic reaction. These and effects of structural features of the B ring noted previously (White & Jeffery, 1973a) clearly contribute to the selection of substrates by the enzyme.
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References