Influence of the Position of the Double Bond in Steroid Substrates on the Efficiency of the Proton-Transfer Reaction by *Pseudomonas testosteroni* 3-Oxo Steroid Δ^4 – Δ^5 -Isomerase

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Studies of the proton-transfer reaction by *Pseudomonas testosteroni* 3-oxo steroid Δ^4 – Δ^5 -isomerase with $\Delta^{5(6)}$ - and $\Delta^{5(10)}$ -steroid substrates demonstrate the importance of the position of the double bond for the efficiency of the isomerization process. Thus 3-oxo- $\Delta^{5(6)}$ -substrates have markedly high k_{cat} values, whereas those of 3-oxo- $\Delta^{5(10)}$ -substrates are very low and their apparent K_m values approach equilibrium dissociation constants. The first step in the isomerization process is:

which is governed by the k_{-1}/k_{+1} ratio and is shown to be very similar for the two classes of substrates (3-oxo- $\Delta^{5(6)}$ - and - $\Delta^{5(10)}$ -steroids). They therefore differ in the steps distal to the initial formation of the Michaelis–Menten complex. The use of the deuterated androst-5(6)-ene-3,17-dione substrate enabled us to calculate individual rate constants k_{+1} and k_{-1} as well as to determine the apparent rate-limiting step in the isomerization process. With the deuterated oestr-5(10)-ene-3,17-dione substrate, no significant isotope effect was observed suggesting that a different rate-limiting step may be operative in this isomerization process. Data are presented that indicate that under optimal concentrations of the efficient androst-5(6)-ene-3,17-dione substrate, the forward reaction for ES complex formation (as defined by k_{+1}) is limited only by diffusion and the apparent K_m does not approach the equilibrium constant, suggesting that the evolution of this enzyme has proceeded close to 'catalytic perfection'.

In a previous paper (Weintraub *et al.*, 1977) we described the structural requirements for ligand recognition for binding by *Pseudomonas testosteroni* 3-oxo steroid Δ^4 – Δ^5 -isomerase (EC 5.3.3.1) in relation to the active site of the enzyme.

Although such kinetic studies with competitive inhibitors can only provide information about the ligand (substrate analogues)-binding step, the subsequent events of the catalytic process cannot be analysed by such an approach. To investigate the different intermediate steps, the conventional approach is to study the kinetics of the catalytic process with various substrates. There are specific difficulties with the isomerase and its classically very efficient substrates, 3-oxo- $\Delta^{5(6)}$ -steroids, owing

to their very high turnover numbers [(0.9–9) $\times 10^6 \text{ min}^{-1}$]. Except for carbonic anhydrase (turnover number $3.6 \times 10^7 \text{ min}^{-1}$), the steroid isomerase has the largest turnover number known in enzymology. With such high k_{cat} values it becomes difficult, if not impossible, even with the most rapid techniques available, to observe some of the expected intermediate complexes. Therefore, investigations have been undertaken with slower substrates, which have been described previously, but without turnover-number data (Kawahara & Talalay, 1960; Kawahara *et al.*, 1962; Nes *et al.*, 1963). Information about the rate-limiting step involved in the proton-transfer process experiments have been obtained with substrates (3-oxo- $\Delta^{5(10)}$ - and - $\Delta^{5(6)}$ -steroids) deuterated at a specific carbon position (4 β). The difference in the isotope effect suggests that not the same rate-limiting step would be

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operative for the $\Delta^{5(6)}$ - or $\Delta^{5(10)}$ -substrate isomerization. These studies also demonstrate the importance of the position and of the number of double bonds in the proton-transfer reaction efficiency, since the calculated k_{cat} values for 3-oxo- $\Delta^{5(6)}$ -substrates range between 0.5×10^5 and $1.4 \times 10^5 \text{ s}^{-1}$, whereas those of 3-oxo- $\Delta^{5(10)}$ -substrates are only in the range $(1.8\text{--}3.6) \times 10^2 \text{ s}^{-1}$. Moreover, potential steroid substrates (e.g. 3-oxo- $\Delta^{5(10),9(11)}$ -steroid) are not all isomerized.

Experimental

Materials

Enzyme. Isomerase was purified by the procedure of Weintraub *et al.* (1973). Homogeneity was checked by the characteristic u.v. fluorescence excitation-emission spectra by polyacrylamide-gel electrophoresis as previously described (Vincent *et al.*, 1976) and by immunodiffusion and immunoelectrophoresis (H. Weintraub, E.-E. Baulieu & A. Alfens, unpublished work).

The specific activity of the isomerase dimer was about 55000 units/mg of enzyme protein, using the new $A_{1\text{cm}}^{0.1\%}$ value of 0.328 ± 0.004 at 280 nm (Weintraub *et al.*, 1973).

Chemicals. Substrates. Unlabelled substrates and inhibitors were given by Roussel Uclaf and found to be >95% pure as verified by t.l.c. on silica gel (F254.Merck) plates (10 cm $\times$ 5 cm). Ascending elution was performed with ethyl acetate/hexane (1:1, v/v) as solvent. Oxo Δ^4 -steroids were identified by examination under a u.v. lamp. Staining was obtained by spraying a 30% H_2SO_4 /water solution and drying at 120°C for 10 min. This technique detected only more than 5% impurities. They were used without further purification.

Deuterated substrates. [$4\beta\text{-}^2\text{H}$]Androst-5(6)-stene-3,17-dione (0.895 atom of ^2H) and [$4\beta\text{-}^2\text{H}$]oestr-5(10)-ene-3,17-dione (0.95 atom of ^2H) were synthesized and kindly provided by Professor A. Marquet. The ^2H content was estimated by mass spectrometry and its localization was checked as previously described (Viger & Marquet, 1977).

Potassium phosphate buffer. This was prepared from Merck products.

Methanol and ethanol were purchased from Prolabo.

Methods

Kinetic measurements. Initial isomerization rates (v_i) were recorded within the first 3 min (at $25 \pm 0.05^\circ\text{C}$) by measuring ΔA_{248} with a Cary 15 recording spectrophotometer as previously described (Weintraub *et al.*, 1977). Addition of either methanol or ethanol, with or without steroid substrates, produced a pH increase that remained, however, small and did not affect kinetic parameters in the pH range studied (Weintraub *et al.*, 1972).

Depending on steroid solubility, substrates and competitive inhibitors were used in the concentration range of 50–750 μM . Isomerase concentrations ranging from 7.5 pM to 75 fM were used, diluted in bovine serum albumin (1%, w/v), which does not affect enzymic parameters and avoids adsorption of the enzyme on the silica cells. The standard reaction cell contained (in a final volume of 3 ml) potassium phosphate buffer (0.03 M), pH 7.0, and the steroid either in methanol or ethanol (3.3–10%, v/v). The reaction was initiated by adding 5–20 μl of the isomerase solution. The control blank cell contained all reactants except the enzyme. Spontaneous isomerization of all substrates was found to be negligible under the experimental conditions used. The apparent K_m (Michaelis constant), K_s (the equilibrium dissociation constant), K_i (the inhibition constant) for competitive inhibitors and k_{cat} (the catalytic constant) were determined from plots by the methods of Lineweaver & Burk (1934) and Dixon (1953). The nature of the inhibition was demonstrated by the method of Dixon (1953).

K_s determination for $\Delta^{5(10)}$ -substrates. When two substrates react with a single enzyme without formation of a ternary complex and when working under experimental conditions, such that the poorer substrate is not transformed at the enzyme concentration employed, the latter may be considered as a competitive inhibitor, and thus the K_i value determined represents its K_s and may be considered as an equilibrium dissociation constant (Dixon & Webb, 1958; Pocklington & Jeffrey, 1969). Accordingly, we studied three $\Delta^{5(10)}$ -steroid substrates, which were slow substrates relative to androst-5(6)-ene-3,17-dione and oestr-5(6)-ene-3,17-dione (which were the rapid substrates used). Under the same experimental conditions as those employed for $\Delta^{5(6)}$ -substrates, a negligible isomerization rate of $\Delta^{5(10)}$ -substrates was observed.

^2H primary isotope effect on the proton transfer of oestr-5(10)-ene-3,17-dione and androst-5(6)-ene-3,17-dione. Studies of the isomerization reaction of the [$4\beta\text{-}^2\text{H}$]labelled and non-labelled oestr-5(10)-ene-3,17-dione and androst-5(6)-ene-3,17-dione substrates were performed under identical experimental conditions: in potassium phosphate buffer (0.03 M), pH 7.0, at 25°C and in either methanol or ethanol (3.3–10%, v/v).

Results

Comparison of kinetic parameters of $\Delta^{5(6)}$ - and $\Delta^{5(10)}$ -substrates

Table 1 shows K_m , K_s and k_{cat} constants for the isomerization of $\Delta^{5(6)}$ - and $\Delta^{5(10)}$ -substrates. The K_m and k_{cat} constants are different for the corresponding $\Delta^{5(6)}$ - and $\Delta^{5(10)}$ -substrates. Comparison

Table 1. K_m , K_s and k_{cat} values for $\Delta^{5(6)}$ and $\Delta^{5(10)}$ -substrates of isomerase at different methanol concentrations
Experiments were done in potassium phosphate buffer (0.03 M), pH 7.0, at 25°C. K_m and k_{cat} values were determined from Lineweaver-Burk plots. K_s values, the equilibrium dissociation constants of $\Delta^{5(10)}$ -substrates considered as inhibitors, were determined by Dixon plots and are expressed in μM ligand concentration. Androst-5(6)-ene-3,17-dione and oestr-5(6)-ene-3,17-dione were used as rapid reference substrates. The standard deviation was $\pm 10\%$ for apparent K_m values and $\pm 3.5\%$ for k_{cat} .

Substrates	Methanol concn. (% v/v)	Kinetic parameters		
		3.3	5.0	10.0
Androst-5(6)-ene-3,17-dione	K_m (μM) k_{cat} (s^{-1})	310 14.10×10^4	357 9.70×10^4	660 8.20×10^4
Oestr-5(6)-ene-3,17-dione	K_m (μM) k_{cat} (s^{-1})	83 5.40×10^4	183 5.60×10^4	450 6.10×10^4
Oestr-5(10)-ene-3,17-dione	K_m (μM) k_{cat} (s^{-1}) K_s (μM)	48 1.25×10^2 50	120 0.95×10^2 117	270 1.10×10^2 265-270
17 β -Hydroxyoestr-5(10)-en-3-one	K_m (μM) k_{cat} (s^{-1}) K_s (μM)	40 0.38×10^2 39	55 0.36×10^2 50	100 0.36×10^2 95
17 α -Ethynyl-17 β -hydroxyoestr-5(10)-en-3-one	K_m (μM) k_{cat} (s^{-1}) K_s (μM)		142 1.83×10^2 140	300 1.78×10^2 295

between $\Delta^{5(10)}$ - and the corresponding $\Delta^{5(6)}$ -substrates gives evidence that the shift of the double bond from C-5(6) to C-5(10) induces only a minor but non-negligible decrease in K_m (2–3-fold), and a very marked decrease in $k_{cat.}$ (up to 600-fold).

The replacement of the 17-oxo group by a 17 β -hydroxy group in estr-5(10)-ene-3,17-dione results in a decrease in $k_{cat.}$ (3-fold) and in a parallel decrease of K_m . Addition of a 17 α -ethynyl substituent to the 17 β -hydroxyestr-5(10)-en-3-one substrate increases the K_m and $k_{cat.}$ values almost to the same extent. It has to be emphasized that the presence of a double bond between C-5 and C-10 modifies the spatial configuration of the substrate molecule and in the isomerization process of $\Delta^{5(10)}$ -substrates the proton acceptor will not be from C-6 as for $\Delta^{5(6)}$ -steroid substrate, but from C-10. This

proton could originate from the 4 β -position either directly or indirectly via proton exchange with the medium.

Proton-transfer reaction of $\Delta^{5(10)}$ -substrates

As shown in Table 1, at two different methanol concentrations (5 and 10%, v/v) when $\Delta^{5(10)}$ -substrates were used as competitive inhibitors (Dixon & Webb, 1958; Pocklington & Jeffrey, 1969), and whatever rapid reference $\Delta^{5(6)}$ -substrates were employed, k_m was found to equal K_s ($\pm 10\%$ for the order of experimental error for kinetic measurements). According to eqn. (1):

Table 2. K_m and $k_{cat.}$ values for the isomerization of androst-5(6)-ene-3,17-dione and the corresponding (4 β - 2 H)-labelled substrate at different concentrations of methanol

$K_{m(^2H)}$ is the Michaelis constant with the (4 β - 2 H)-labelled substrate, $K_{m(H)}$ is the Michaelis constant with the non-labelled substrate, $k_{cat.(^2H)}$ is the catalytic constant for the (4 β - 2 H)-labelled substrate and $k_{cat.(H)}$ is the catalytic constant for the non-labelled substrate. Standard deviation was $\pm 10\%$ for the apparent K_m and $\pm 3.5\%$ for the $k_{cat.}$.

Methanol concn. (% v/v) ...	Kinetic parameters		
	3.3	5	10
Non-labelled substrate	$K_{m(H)} = 310 \mu M$ $k_{cat.(H)} = 14.1 \times 10^4 s^{-1}$	$K_{m(H)} = 357 \mu M$ $k_{cat.(H)} = 9.70 \times 10^4 s^{-1}$	$K_{m(H)} = 660 \mu M$ $k_{cat.(H)} = 8.20 \times 10^4 s^{-1}$
(4 β - 2 H)-labelled substrate	$K_{m(^2H)} = 170 \mu M$ $k_{cat.(^2H)} = 2.70 \times 10^4 s^{-1}$	$K_{m(^2H)} = 200 \mu M$ $k_{cat.(^2H)} = 1.50 \times 10^4 s^{-1}$	$K_{m(^2H)} = 250 \mu M$ $k_{cat.(^2H)} = 0.847 \times 10^4 s^{-1}$
$K_{m(H)}/K_{m(^2H)}$	1.80	1.80	2.40
$k_{cat.(H)}/k_{cat.(^2H)}$	5.20	6.60	9.70
Efficiency coefficient ($k_{cat.(H)}/K_{m(H)}$)	$4.55 \times 10^8 M^{-1} \cdot s^{-1}$	$2.70 \times 10^8 M^{-1} \cdot s^{-1}$	$1.24 \times 10^8 M^{-1} \cdot s^{-1}$
Efficiency coefficient ($k_{cat.(^2H)}/K_{m(^2H)}$)	$1.59 \times 10^8 M^{-1} \cdot s^{-1}$	$0.75 \times 10^8 M^{-1} \cdot s^{-1}$	$0.34 \times 10^8 M^{-1} \cdot s^{-1}$

Table 3. K_m and $k_{cat.}$ values for the isomerization of the androst-5(6)-ene-3,17-dione and the corresponding (4 β - 2 H)-labelled substrate at different ethanol concentrations

The standard deviation was $\pm 10\%$ for the apparent K_m and $\pm 3.5\%$ for the $k_{cat.}$.

Ethanol concn. (% v/v) ...	Kinetic parameters		
	3.3	5	10
Non-labelled substrate	$K_{m(H)} = 200 \mu M$ $k_{cat.(H)} = 7.70 \times 10^4 s^{-1}$	$K_{m(H)} = 497 \mu M$ $k_{cat.(H)} = 6.80 \times 10^4 s^{-1}$	$K_{m(H)} = 1250 \mu M$ $k_{cat.(H)} = 5.70 \times 10^4 s^{-1}$
(4 β - 2 H)-labelled substrate	$K_{m(^2H)} = 125 \mu M$ $k_{cat.(^2H)} = 1.50 \times 10^4 s^{-1}$	$K_{m(^2H)} = 200 \mu M$ $k_{cat.(^2H)} = 1.00 \times 10^4 s^{-1}$	$K_{m(^2H)} = 550 \mu M$ $k_{cat.(^2H)} = 0.60 \times 10^4 s^{-1}$
$K_{m(H)}/K_{m(^2H)}$	1.60	2.10	2.30
$k_{cat.(H)}/k_{cat.(^2H)}$	5.00	7.00	9.40
Efficiency coefficient ($k_{cat.(H)}/K_{m(H)}$)	$3.85 \times 10^8 M^{-1} \cdot s^{-1}$	$1.37 \times 10^8 M^{-1} \cdot s^{-1}$	$0.456 \times 10^8 M^{-1} \cdot s^{-1}$
Efficiency coefficient ($k_{cat.(^2H)}/K_{m(^2H)}$)	$1.20 \times 10^8 M^{-1} \cdot s^{-1}$	$0.50 \times 10^8 M^{-1} \cdot s^{-1}$	$0.109 \times 10^8 M^{-1} \cdot s^{-1}$

with $\Delta^{5(10)}$ -substrates, $k_{\text{cat.}}$ must be negligible compared with k_{-1} . Conversely, for the isomerization of androst-5(6)-ene-3,17-dione, the apparent Michaelis constant (K_m) never approaches the equilibrium dissociation constant, since it differs from the ratio k_{-1}/k_{+1} (K_s) (see Tables 2, 3, 4 and 5).

Primary isotope effect on kinetic parameters for isomerization of androst-5(6)-ene-3,17-dione

Tables 2 and 3 give the $k_{\text{cat.}}$ and K_m constants for the $[4\beta\text{-}^2\text{H}]$ androst-5(6)-ene-3,17-dione ($K_{m(^2\text{H})}$ and $k_{\text{cat.}(^2\text{H})}$) and the corresponding unlabelled substrate ($K_{m(\text{H})}$ and $k_{\text{cat.}(\text{H})}$), in methanol or ethanol at different concentrations, under the same experimental conditions. The $k_{\text{cat.}}$ value of the ^2H -labelled substrate is markedly decreased compared with the non-labelled substrate, whereas the K_m is only slightly affected. Such an isotope effect on $k_{\text{cat.}} > 5$, according to Richards (1970), Northrop (1975), Jencks (1975) and Alberty & Knowles (1976a), indicates that the rate-limiting step involves cleavage of the C-H bond concerned. Furthermore, this isotope effect is strongly enhanced as methanol or ethanol concentrations are increased. From the large isotope effect observed, it is possible to calculate the rate constants k_{+1} and k_{-1} , with the assumption of a simplified catalytic process reduced to the classical Michaelis-Menten equation (eqn. 1) for the isomerization reaction, and an irreversible product formation, which was observed with $\Delta^{5(6)}$ -substrates (Malhotra & Ringold, 1965; Falcoz-Kelly *et al.*, 1968).

It follows that:

$$K_{m(^2\text{H})} = \frac{k_{-1}}{k_{+1}} + \frac{k_{\text{cat.}(^2\text{H})}}{k_{+1}}$$

$$K_{m(\text{H})} = \frac{k_{-1}}{k_{+1}} + \frac{k_{\text{cat.}(\text{H})}}{k_{+1}}$$

$$k_{+1} = \frac{k_{\text{cat.}(\text{H})} - k_{\text{cat.}(^2\text{H})}}{K_{m(\text{H})} - K_{m(^2\text{H})}}$$

$$k_{-1} = K_{m(\text{H})}k_{+1} - k_{\text{cat.}(\text{H})}$$

$$k_{-1} = K_{m(^2\text{H})}k_{+1} - k_{\text{cat.}(^2\text{H})}$$

It should, however, be emphasized that without the assumption of the identity of k_{+1} and k_{-1} for the ^2H -labelled and non-labelled substrates, which necessarily implies that there is no change in the Michaelis-Menten complex formation due to substitution of ^2H for H, the calculation of these rate constants would be impossible (Simon & Palm, 1966).

Tables 4 and 5 give the individual rate constants k_{+1} and k_{-1} and the efficiency parameters $k_{\text{cat.}}/K_m$ calculated (at different concentrations of methanol and ethanol) for the isomerization of androst-5(6)-ene-3,17-dione. It is seen that as the concentration of either methanol or ethanol is increased, the rate constant k_{+1} decreases from 8×10^8 to $0.7 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ and since k_{-1} decreases from 11×10^{-4} to $5 \times 10^{-4} \text{ M}^{-1} \cdot \text{s}^{-1}$, the k_{-1}/k_{+1} ratio is increased, resulting in a decrease in the efficiency parameter. The observations of the effect of increasing co-solvent concentrations on the k_{-1}/k_{+1} ratio is in agreement with the previously reported results with competitive inhibitors (Weintraub *et al.*, 1972; Jones & Gordon, 1973).

$\Delta^{5(10)}$ -Substrates

No significant isotope effect (< 2) was detected on the isomerization rate of deuterated oestr-5(10)-ene-3,17-dione substrate. Accordingly, it was not possible to calculate k_{+1} and k_{-1} .

Table 4. Calculated rate constants k_{+1} and k_{-1} for the isomerization of androst-5(6)-ene-3,17-dione at different concentrations of methanol

Methanol concn. (%, v/v)	$10^{-8} \times k_{+1} (\text{M}^{-1} \cdot \text{s}^{-1})$	$10^{-4} \times k_{-1} (\text{s}^{-1})$	$k_{-1}/k_{+1} = K_s (\mu\text{M})$	$K_{m(\text{H})} (\mu\text{M})$
3.3	8.1	11.1	140	310
5.0	5.6	9.8	170	359
10.0	2.0	5.1	240	660

Table 5. Calculated rate constants k_{+1} and k_{-1} for the isomerization of androst-5(6)-ene-3,17-dione at different concentrations of ethanol

Ethanol concn. (%, v/v)	$10^{-8} \times k_{+1} (\text{M}^{-1} \cdot \text{s}^{-1})$	$10^{-4} \times k_{-1} (\text{s}^{-1})$	$k_{-1}/k_{+1} = K_s (\mu\text{M})$	$K_{m(\text{H})} (\mu\text{M})$
3.3	8.2	8.1	106	200
5.0	2.7	3.8	744	417
10.0	0.7	3.4	467	1250

Table 6. *Inhibition constants (K_i) of three competitive inhibitors*

Experiments were done at 25°C in potassium phosphate buffer (0.03 M), pH 7.0, in 10% (v/v) methanol and at four different concentrations of androst-5(6)-ene-3,17-dione used as the substrate. K_i values are expressed as μM concentrations and were determined by Dixon plots.

The importance of the position of the double bond in substrates for the enzymic proton-transfer reaction

In an attempt to investigate the influence of the number and the position of double bonds in substrates, 3-oxo- $\Delta^{5(10)}$ -compounds with a supplementary conjugated $\Delta^{9(11)}$ -double bond were studied. Interestingly, the resulting $\Delta^{5(10),9(11)}$ -steroids could not be isomerized (Table 6). Despite a 10^4 -fold higher isomerase concentration relative to that used for the isomerization of $\Delta^{5(10)}$ -substrates, no proton transfer could be detected. Table 6 shows also that these 3-oxo- $\Delta^{5(10),9(11)}$ -steroids 'potential substrates' are relatively high-affinity competitive inhibitors. The 17β-hydroxyoestra-5(10),9(11)-dien-3-one and the corresponding $\Delta^{4(5),9(10)}$ -isomer, which is a competitive inhibitor, have similar K_i values.

Has the isomerase succeeded in evolving almost to catalytic perfection?

The concept that an enzyme has evolved almost to catalytic perfection has been described by Brocklehurst & Cornish-Bowden (1976).

Considering the isomerase from this point of view with its most efficient substrate androst-5(6)-ene-3,17-dione, it is seen (Tables 4 and 5) that the k_{cat} value is very high and that the K_m differs from the K_s (k_{-1}/k_{+1}) and generally approaches a value of $3K_s$. At low co-solvent concentrations (3.3%, v/v), the efficiency parameter k_{cat}/K_m approaches $5 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ in methanol and $3.8 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ in ethanol. These results are consistent with the view that the isomerase has succeeded in evolving almost to catalytic perfection. It is important to emphasize that 'catalytic perfection' with respect to the isomerase is well illustrated with its 'perfect natural substrate', whereas with the inefficient oestr-5(10)-ene substrates and under the same experimental conditions, the apparent K_m approaches the equilibrium

dissociation constant and k_{cat}/K_m equals $2.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, a value that is far less than the limit observed with the androst-5(6)-ene-3,17-dione substrate.

Discussion

The slower enzymic isomerization rate of 3-oxo- $\Delta^{5(10)}$ -substrates relative to the $\Delta^{5(6)}$ -substrates has previously been reported (Kawahara & Talalay, 1960; Nes *et al.*, 1963), but initial isomerization rates, relative to androst-5(6)-ene-3,17-dione, were determined at only a single $\Delta^{5(10)}$ -substrate concentration, and the isomerase employed was a crude preparation. Kawahara *et al.* (1962) have also reported K_m values with the 17β-hydroxyoestra-5(10)-ene-3-one at a single low methanol concentration (3.3%, v/v), but only V_{max} values relative to androst-5(6)-ene-3,17-dione were given.

In the present study, we have compared 3-oxo- $\Delta^{5(10)}$ - and - $\Delta^{5(6)}$ -steroid substrates at a wide range of substrate concentrations with two co-solvent systems and a homogeneous crystalline isomerase preparation. It was found that the position of the double bond has a drastic influence on the proton-transfer reaction.

Thus, $\Delta^{5(6)}$ -substrates have markedly high k_{cat} values, relative to the corresponding $\Delta^{5(10)}$ -steroid substrates. Under identical experimental conditions 3-oxo- $\Delta^{5(6)}$ - and - $\Delta^{5(10)}$ -steroid substrates have K_m values of the same order of magnitude. Unexpectedly, it was observed for the first time that the addition of the supplementary conjugated $\Delta^{9(11)}$ -double bond to a 3-oxo- $\Delta^{5(10)}$ -substrate removes the possibility of isomerization (Table 6); however, these latter polyunsaturated steroids are competitive inhibitors with relatively high affinity for the isomerase. Their K_i values are significantly lower than the K_m values of substrates (see Tables 1 and 6).

With deuterated substrates, no significant isotope effect on the K_m and k_{cat} parameters could be detected with the 3-oxo- $\Delta^{5(10)}$ -substrate. Furthermore, it has been demonstrated (Viger & Marquet, 1977; Geynet, 1975) that only 60% of the proton transfer is intramolecular and 40% 4β -protons are exchanged with the medium. On the other hand, a large primary isotope effect on k_{cat} and a negligible effect on K_m was observed with androst-5(6)-ene-3,17-dione. This isotope effect is markedly enhanced with increasing co-solvent concentration. Such an observation confirms the participation of solvent in the proton-transfer reaction as previously reported (Weintraub *et al.*, 1972) and definitely precludes an entirely intramolecular proton-transfer process (Malhotra & Ringold, 1965) in agreement with the experimental data of Viger & Marquet (1977), which give a 70% intramolecular proton transfer and 30% of proton exchange with the medium.

From the calculated k_{-1}/k_{+1} ratio, it was concluded that one cannot neglect the k_{cat} parameter compared with k_{-1} , and therefore the apparent K_m

$$\left(K_m = \frac{k_{-1} + k_{cat.}}{k_{+1}} \right)$$

with such a $\Delta^{5(6)}$ -steroid substrate does not approach the equilibrium constant, whereas, with 3-oxo- $\Delta^{5(10)}$ -substrates, it was found that the K_m and the K_s values determined were equal. In summary, these results demonstrate the striking difference in the isomerization process between the two classes of substrates 3-oxo- $\Delta^{5(6)}$ - and - $\Delta^{5(10)}$ -steroids.

To analyse the different kinetic behaviour of $\Delta^{5(6)}$ - and $\Delta^{5(10)}$ -substrates, the pathway described by scheme (1) has been applied to the proton-transfer reaction for the steroid isomerase. The first step i.e. the enzyme-substrate complex formation, governed by the k_{-1}/k_{+1} ratio, is not very different for the two classes of isomerase substrates. Indeed, the k_{-1}/k_{+1} ratio for the oestr-5(10)-ene-3,17-dione is experimentally obtained by the K_m value, which

approaches the equilibrium dissociation constant (K_s), whereas with the androst-5(6)-ene-3,17-dione substrate, this k_{-1}/k_{+1} ratio has been calculated from experimental data with the deuterated substrate (Tables 4 and 5). For instance at 10% (v/v) methanol as a co-solvent (Tables 1 and 3) the K_m values of oestr-5(10)-ene-3,17-dione and of androst-5(6)-ene-3,17-dione are almost the same (0.27 and 0.24 mM respectively). For the slower $\Delta^{5(10)}$ -substrate, k_{cat} equals $1.1 \times 10^2 \text{ s}^{-1}$, whereas the k_{cat} of the rapid substrate is $8.2 \times 10^4 \text{ s}^{-1}$. Therefore with oestr-5(10)-ene-3,17-dione:

$$\frac{(k_{-1} + k_{cat.})}{k_{+1}} = 0.27 \text{ mM}$$

or

$$\frac{k_{-1} + 1.1 \times 10^2 \text{ s}^{-1}}{k_{+1}} = 0.27 \text{ mM}$$

To fulfil these conditions, the k_{cat} must be negligible compared with k_{-1} ($K_s = K_m$) and the smallest value of k_{-1} has to be at least 10^3 s^{-1} ; then:

$$\frac{10^3 \text{ s}^{-1} + 1.1 \times 10^2 \text{ s}^{-1}}{k_{+1}} = 0.27 \text{ mM}$$

and under these conditions:

$$\begin{aligned} k_{+1} &= \frac{(10 \cdot 10^2 + 1.1 \times 10^2) \text{ s}^{-1}}{2.7 \times 10^{-4} \text{ M}} \\ &= 4.07 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \end{aligned}$$

Comparing this value with the k_{+1} of the androst-5(6)-ene-3,17-dione ($2 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$; Table 4) it is seen that they differ only by a factor of 50, whereas there is a 740-fold difference in k_{cat} constants. The above calculation was made taking the lowest k_{-1} value, consistent with the negligible k_{cat} value resulting from $K_m = K_s$; if, however, one assumes a higher value for k_{-1} (10^4 s^{-1}), then k_{+1} equals $3.7 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ and k_{+1} differs for both substrates only by a factor of 5.4. On this latter assumption the k_{-1} value has the same order of magnitude for both substrates. At present the individual rate constants for $\Delta^{5(10)}$ -substrates are unknown; the stopped-flow technique with slow substrates should give the required information.

Considering the results presented, it should be emphasized that the two substrates studied were not totally identical. Only the $[4\beta\text{-}^2\text{H}]$ androst-5(6)-ene-3,17-dione with an angular 19-methyl group was available. Despite this, it seems most unlikely that the high efficiency of the $\Delta^{5(6)}$ -substrate would only result from the higher association constant k_{+1} for the formation of the Michaelis complex, since the non-labelled androst-5(6)-ene-3,17-dione and oestr-5(6)-ene-3,17-dione have under identical conditions almost the same kinetic parameters (Table 1).

Scheme 1. Pathway for isomerization process, in which a reaction intermediate (EZ) can exchange deuterium with solvent

Unprimed rate constants refer to the all-hydrogen system. Primed rate constants indicate substitution of unlabelled hydrogen by ^2H . P is the reaction product; EZ' is the reaction intermediate, which can exchange ^2H with the solvent.

Fig. 1. Proton-transfer reaction of oestr-5(6)- and oestr-5(10)-ene-3,17-dione substrates by the isomerase giving the same reaction product

As shown in the present work, the ES complex is most unlikely to be different for the two classes of substrates. It was also shown that EP reactants for $\Delta^{5(6)}$ - and $\Delta^{5(10)}$ -substrates are identical (Fig. 1). Therefore one should expect identical energy states from the EP to the E + P step (Scheme 1) excluding the possibility of this step as rate limiting for the $\Delta^{5(10)}$ proton-transfer reaction. The different results obtained with the ^2H -labelled $\Delta^{5(6)}$ - and $\Delta^{5(10)}$ -substrates therefore arise necessarily from differences in the internal states from ES to EP (Scheme 1).

The large decrease in the enzyme-catalysed C-4 $\rightarrow$ C-6 proton-transfer reaction resulting from the H- ^2H substitution at the C-4 β position in the androst-5(6)-ene-3,17-dione substrate has been interpreted as identifying the C-4 β hydrogen-bond cleavage or C-6 β reprotonation as the rate-limiting step. The absence of any significant isotope effect on the C-4 $\rightarrow$ C-10 proton-transfer reaction observed with the deuterated oestr-5(10)-ene-3,17-dione is now interpreted as indicating that the rate-limiting step in the isomerization process does not involve either C-H bond cleavage or the C-10 reprotonation. However, this conclusion rests on the assumption that neither the product nor the substrate rapidly exchange ^2H with solvent. Alberly & Knowles (1976a) have demonstrated that under certain conditions the enzyme-bound intermediate (EZ' in Scheme 1) exchanges ^2H with solvent. This

exchange has been termed a 'washing-out effect' (Leadlay *et al.*, 1976). Such exchange occurs in the isomerase system with the $\Delta^{5(10)}$ -substrate at a limited rate (40%) (Geynet, 1975). Accordingly, the fact that no significant isotope effect was observed with [4 β - ^2H]oestr-5(10)-ene-3,17-dione strongly suggests that the rate-limiting steps for both classes of substrates are really different.

Sih & Whitlock (1968) have reported an absence of any isotope effect for the isomerization reaction of the [4,4'- ^2H]pregn-5(6)-ene-3,20-dione and a 100-fold slower dissociation rate for the EP complex relative to the values with androst-5(6)-ene-3,17-dione. Accordingly, these authors have suggested that product release from the EP complex and not C-H bond cleavage might well be the rate-limiting step in the isomerization reaction of pregn-5(6)-ene-3,17-dione. Unfortunately, the complete details of this preliminary study have not been published. However, there may be a rapid 'washing out effect' of ^2H with the later substrate before C- ^2H bond cleavage, which would lead to the absence of any isotope effect. Our present working hypothesis may be summarized as follows. The large k_{cat} differences observed between $\Delta^{5(6)}$ - and $\Delta^{5(10)}$ -substrates arise from a different intermediate rate-limiting step subsequent to the ES complex formation. $\Delta^{5(10)}$ -substrate molecules have different spatial configurations compared with $\Delta^{5(6)}$ -steroids. Whereas with $\Delta^{5(6)}$ -substrates one may assume a perfect fit

Fig. 2. Partially intramolecular $4\beta \rightarrow 6\beta$ proton transfer for the classical androst-5(6)-ene-3,17-dione substrate by the isomerase via an enol (EZ) intermediate complex as proposed by Ringold & Malhotra (1965) and Viger & Marquet (1977)

ES is the Michaelis complex, EZ is the enol-intermediate complex and EP is the enzyme-product complex before product release. A is a proton donor and B is a proton acceptor.

between the bifunctional acid-basic groups of the active site of the isomerase (Fig. 2) and the substrate area involved in the proton-transfer reaction, with $\Delta^{5(10)}$ -substrates, on the other hand, such a substrate-enzyme-induced fit is less perfect, so that there is a higher energy requirement to achieve the appropriate fit of the active site and the steroid substrate, resulting in a slowing down of the overall reaction rate.

The fact that 3-oxo- $\Delta^{5(10),9(11)}$ -compounds, which are rigid molecules owing to the presence of a supplementary conjugated double bond, are ineffective substrates supports the view that the correct orientation for C-H bond cleavage is energetically impossible with such steroids, despite a strong binding to the isomerase. It follows, therefore, that binding recognition by the enzyme active site of steroid with structural requirements of a steroid substrate is necessary but not sufficient for isomerization to occur.

It is of interest to consider whether the isomerase has evolved to fit the concept of catalytic perfection as proposed by Brocklehurst & Cornish-Bowden (1976). Improvement to achieve catalytic perfection could arise from an increase in k_{cat} , but in relation to k_{-1} , and only when $k_{\text{cat}} > k_{-1}$, can k_{cat}/K_m , the second-order rate constant for the overall reaction, approach the diffusion limit; therefore for a highly efficient enzyme one may expect K_s to be less than K_m . Isomerase with the efficient natural androst-5(6)-ene-3,17-dione substrate has the properties of such enzymes.

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