ELECTRONIC CONTROL OF ENZYMATIC OXIDATION-REDUCTION PROCESSES. THE REDUCTION OF Δ^4-3-KETOSTEROIDS TO Δ^4-3-OLS BY THE 3α- AND 3β-HYDROXYSTEROID DEHYDROGENASES OF PSEUDOMONAS TESTOSTERONI[1].

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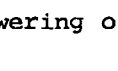
Received August 24, 1963

Ringold, Ramachandran and Forchielli (1962, 1963) noted that certain halogenated Δ^4-3-ketosteroids (2α-fluoro,6β-fluoro,6α-fluoro, 4-chloro) in contrast to unsubstituted Δ^4-3-ketones, are reduced to allyl alcohols (Δ^4-3-hydroxy compounds) by DPNH or TPNH in the presence of crude male rat liver supernatant fractions It was proposed (Ringold et.al., 1962, 1963) that allyl alcohol formation is a reduction catalyzed by the saturated steroid 3-hydroxy dehydrogenases known to be present in this fraction and that non-halogenated Δ^4-3-ketones are not measurably reduced to Δ^4-3-ols because of their greater resonance stabilization relative to saturated 3-ketones. The facile reduction in the presence of a halogen substituent was attributed to destabilization of the

[1]Supported in part by grants A-4044 and CY-4550, National Institute of Health.

unsaturated ketone moiety in the ground state by inductive effect[2] and stabilization of an anionic intermediate in the transition state (e.g. ) by charge delocalization, the net effect being a lowering of the free energy of activation in the presumed rate-determining hydride transfer step. The multiplicity of enzymes in the rat liver fraction left this supposition in the realm of an attractive hypothesis however and the study of a less complex enzyme system has been desirable.

The DPN dependent adaptive 3α- and 3β-hydroxysteroid dehydrogenases of pseudomonas testosteroni (Talalay, 1960 and earlier references therein) have proven to be such a system. We report herein that a cell-free preparation of this organism which contains these two enzymes and which is ordinarily considered to reduce saturated but not α,β-unsaturated 3-ketosteroids, readily effects the reduction of the aforementioned halogenated steroids to a mixture of Δ^4-3α- and Δ^4-3β-alcohols. Initial rates of reduction at pH 6.7 were measured spectrophotometrically by the disappearance of the 340 mμ maximum of DPNH and are listed in table I for steroid concentrations of ca. 1.3×10^{-4} M.[3] A saturated 3-ketosteroid, 17β-hydroxy-17α-methyl-5α-androstan-3-one (17αMeDHT), has been included for comparison and it may be noted that the fastest of the fluoro derivatives, 2α-fluorotestosterone,

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[2] In the case of C-2 or C-4 halogen substitution, halogen-carbonyl oxygen dipole-dipole repulsion is also an important destabilizing factor.

[3] Comparable results were obtained at substrate concentrations of ca. 0.3×10^{-4} M and 0.6×10^{-4} M.

reduces at 70% of the rate of the saturated ketone. Allyl alcohol formation was established in each case by direct comparison with authentic standards utilizing thin-layer chromatography on silica gel (benzene-ethyl acetate system). With the exception of 4-fluor and 6β-fluorotestosterone, which appeared to yield almost equal mixtures of the 3α- and 3β-hydroxy derivatives, the 3α-hydroxy isomer predominated.[4]

Table I

Relative rates of reduction of the 3-keto group of testosterone derivatives by adaptive enzymes from pseudomonas testosteroni

Steroid	Relative Rate	Product Formed Δ^4-3α-ol	Δ^4-3β-o
17α-Methyl DHT	21		
2αF testosterone	13	+++	+
6βF testosterone	7	++	++
4F testosterone	6	++	++
4Cl testosterone	4	+++	+
6αF testosterone	3	+++	+
Testosterone	1	+	+
2αMe testosterone	< 1	+	?
6βMe testosterone	< 1	+	?

In a 3 ml. cuvette, solution A (1.0 ml.) (see experimental) was admixed with 1.5 ml. of .03 M phosphate buffer (pH 6.0), and DPNH (400 μg.) contained in .05 ml. of .03 M phosphate buffer (pH 7.2 was added. The steroid (100 μg.) in .05 ml. of ethanol was then added and the disappearance of DPNH at 340 mμ measured. Rates a reported for less than 20% reduction. The pH of the final react mixture was 6.7.

[4]The 3α-hydroxysteroid dehydrogenase is present in predominant quantities in this unfractionated enzyme preparation (see Talalay).

It was noted that at pH 6.7 testosterone itself also underwent reduction, but at an initial rate of only one-third that of the slowest of the halogenated steroids, and formed in extremely low yield a mixture of the known 3α- and 3β-Δ^4-3-ols with the former predominating. In contrast to the halo-derivatives, the extent of reduction was negligible and rapidly reached a point where the oxidation of DPNH was no greater than control values. Only by operating at a lower pH, 5.7, where the equilibrium ratio $\frac{\Delta^4\text{-3-ol}}{\Delta^4\text{-3-one}}$ should undergo approximately a ten-fold shift was it possible to isolate the allyl alcohol. Preparative incubations (described below) of 2α-fluorotestosterone and of testosterone are illustrative of this point. 2α-Methyl- and 6β-methyl-testosterone were also found to undergo reduction to allyl alcohols but at rates even slower than testosterone which is in accord with the electron-releasing effect of a methyl group.

Experiments performed at pH 6.7 with excess DPN instead of DPNH demonstrated that the reverse reaction, the oxidation of Δ^4-3-ol to Δ^4-3-one, proceeded much more rapidly and to a greater extent when no halogen substituent was present. From these oxidation experiments and from the aforementioned reductive experiments it is apparent that halogen substitution leads to a large increase in the equilibrium ratio $\frac{\Delta^4\text{-3-ol}}{\Delta^4\text{-3-one}}$, which is a reflection of the thermodynamic stability of the respective substances. Thus both the rate and the extent of the described enzymatic formation of allyl alcohols are related to the degree of electronic modification of the steroid and are qualitatively predictable on the basis of a simple transition state model.

Preliminary determinations with purified 3α-hydroxydehydrogenase indicate that 2α-fluoro substitution leads to a 150 fold increase in the equilibrium ratio $\frac{\Delta^4\text{-3}\alpha\text{-ol}}{\Delta^4\text{-3-one}}$. Further studies are in progress to determine individual rate and equilibrium constant

Experimental:

Washed and dried whole cells of Pseudomonas testosteroni, adapted by growth in the presence of testosterone according to the procedure of Talalay (1960), were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. One gram of cell preparation was suspended in 40 ml. of .03 M phosphate buffer, pH 7.2, which was also .001 M in ethylenediamine tetra-acetate. The mixture was subjected to sonic disruption for 15 min. in a 10 KC Raytheon Sonic Oscillator, then diluted to 1 l. with the same buffer solution and centrifuged at 7500 x g. for 30 min. The clear supernatant, which contained .04 mg. protein/ml., is designated as solution A.

Solution A (300 ml.) was diluted with 450 ml. of .03 M phosphate buffer (pH 6.0) and 2α-fluorotestosterone (30 mg.) in ethanol (15 ml.) was added, followed by 120 mg. of solid DPNH. The solution was stirred for 30 min. at 27°, the steroids then isolated by extraction with ethyl-acetate and separated by thin-layer chromatography on silica gel using 55% benzene- 45% ethyl acetate. Elution of the major zones followed by crystallization gave 1 mg. of starting material, 2.7 mg. of 2α-fluoro-androst-4-ene-3β,17β-diol, m.p. 180-181° and 16.4 mg. of 2α-fluoro-androst-4-ene-3α,17β-diol, m.p. 238°.

The reduction of 30 mg. of testosterone was carried out in an identical manner but solution A was mixed with .03 M acetate buffer at pH 4.5 giving a final pH of 5.7. Recovered testosterone (22.3 mg.), androst-4-ene-3β,17β-diol, m.p. 155-157° (1.3 mg.) and androst-4-ene-3α,17β-diol, m.p. 225-227° (3.1 mg.) were isolated.

The products of reduction were compared with authentic specimens by melting point admixture and by infrared spectrum in each case.

Acknowledgement

The authors wish to thank Prof. Paul Talalay for confirming the reduction of testosterone to the Δ^4-3α-ol with highly purified 3α-hydroxy dehydrogenase.

References

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