ENZYMIC AND BIOMIMETIC OXIDATIVE COUPLING OF OESTROGENS

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1. Introduction

Peroxidases of diverse origins, including those of the rat uterus [1,2] and of an induced mammary tumour [3], catalyse the transformation of oestrogens. The nature of these transformations received little attention except for the horseradish peroxidase-catalysed conversion [4] of oestradiol into its presumptive tetramer identical with the product obtained by the action of potassium ferricyanide, a reagent known to simulate the biosynthetic oxidative coupling of plant phenols [5,6]. It is now reported that the same transformation is brought about by lactoperoxidase and that oestrone and oestradiol 17-monoacetate undergo analogous transformations. Evidence is presented concerning the site of the reaction and the polymeric character of the products.

2. Experimental

2.1. *General*

Melting points were determined on a Kofler hotstage; those of oestrogen oxidation products in evacuated capillary tubes. Unless otherwise indicated, optical rotations were taken in dioxan, ultraviolet (u.v.) spectra in ethanol–dioxan (49:1, v/v) and infrared (i.r.) spectra in potassium bromide. Horseradish peroxidase (HRP) was supplied by British Drug Houses, Poole, England; lactoperoxidase by Boehringer Corp. (London); radioisotopically-labelled compounds by the Radiochemical Centre, Amersham.

2.2. *Lactoperoxidase-catalysed oxidation of oestradiol*

Oestradiol (150 mg) in *iso*-propanol (25 ml) was added to a stirred mixture of 0.1 M phosphate buffer (1800 ml, pH 7), *iso*-propanol (25 ml) and Triton X-100 (1.8 ml), at 39°C. Lactoperoxidase (5 mg) was added and then, dropwise over a period of 30 min, H_2O_2 (20 ml; 0.6%, v/v). After 16 h at room temperature the formed precipitate was filtered off and then washed well with water. A solution of the crude product (85 mg) in dioxan (1 ml) was dropped into stirred ether (10 ml). The dull-yellow precipitate (30 mg) had an i.r. spectrum identical with that of the oestradiol polymer [4]. After incipient precipitation from acetone the product had m.p. (dec.) 305–310°C undepressed on admixture with authentic material, $[\alpha]_D$ + 68.5° λ_{max} 280 nm (E_1^1 132) and λ_{min} 270 nm (E_1^1 120).

Found: C, 77.7; H, 8.2%.

Calc. $(C_{18}H_{22}O_2)_n \cdot \frac{n}{2}(H_2O)$: C, 77.4; H, 8.3%.

Calc. $(C_{18}H_{22}O_{2.5})_n$: C, 77.7; H, 8.0%.

2.3. *Ferricyanide oxidation of oestrone*

Oestrone was treated as oestradiol [4] with 2.5 mol. equiv. reagent, in a heterogeneous system (benzene–water), under nitrogen. The product eluted with benzene–chloroform (1:1, v/v) from a column of alkaline alumina (Camag, activity I) was obtained in 65–75% yield. Incipient precipitation from benzene–ethanol gave a dull-yellow powder. Its m.p. (dec.) varied with particle size and with the rate of heating; thus, in one instance m.p. values of 278–282°C and 305–310°C were recorded for the same sample. It had $[\alpha]_D$ + 137°, an inflection at 277 nm (E_1^1 135), ν_{max} 1730 cm^{-1} (17-one) and 1450 cm^{-1} (16-CH_2), τ (220 MHz; in deuterochloroform) 2.85 and 3.30 (arom. H), 9.12 (18-CH_3). Mol. wt by vapour pressure osmometry: 2205 in chloroform, 1140 in dioxan; by Rast's method: 1043.

Found: C, 77.8; H, 7.5%.

Calc. $(C_{18}H_{20}O_2)_n \cdot \frac{n}{2}(H_2O)$: C, 77.95; H, 7.6%.

Calc. $(C_{18}H_{20}O_{2.5})_n$: C, 78.2; H, 7.3%.

In ambient air the dried product gained approx. 3% weight in 5–8 h and lost that amount on redrying. No reaction occurred on treatment of the oxidation product with acetic anhydride in pyridine nor on its treatment with diazomethane (exp. performed by D. E. Kime).

Approx. 2.3 mol. equiv. reagent were consumed (out of 4 mol. equiv. available) in the oxidation as determined by iodometric titration in the presence of zinc acetate. With 1 mol. equiv. reagent, the same product was formed in 30–35% yield and oestrone, eluted from the alumina column with ethyl acetate–methanol (1:1. v/v), was recovered in 40–45% yield.

2.4. *Ferricyanide oxidation of radioisotopically labelled oestrone*

[2,4,6α,7α-^{3}H]Oestrone, [6α,7α-^{3}H]oestrone and [6α,9-^{3}H]oestrone (prepared from its potassium sulphate by hydrolysis with acid) were severally admixed with [4-^{14}C]oestrone and with the inert compound and each mixture was then crystallised from 95% ethanol to a constant isotope ratio. [4-^{14}C][2,4,6α,7α-^{3}H]Oestrone (1026 ^{14}C and 8250 ^{3}H dpm/mg) was oxidised with 1 mol. equiv. and 4 mol. equiv. reagent. [4-^{14}C][6α,7α-^{3}H]Oestrone (1034 ^{14}C and 4940 ^{3}H dpm/mg) and [4-^{14}C]-[6α,9-^{3}H]oestrone (29 000 ^{14}C and 89 000 ^{3}H dpm/mg) were oxidised with 1 mol. equiv. and 2.5 mol. equiv. reagent, respectively. The product of each oxidation was isolated as outlined above and was then purified to a constant isotope ratio by incipient precipitation from benzene–ethanol. In reactions with 1 mol. equiv. reagent, the unreacted oestrone was recovered and crystallised from 95% ethanol to a constant isotope ratio.

2.5. *Bromination of [4-^{14}C][2,4,6α,7α-^{3}H]oestrone*

The labelled oestrone (1026 ^{14}C and 8250 ^{3}H dpm/mg) was brominated with 2 mol. equiv. *N*-bromosuccinimide in ethanol [7,8]. The formed [4-^{14}C][6α,7α-^{3}H]2,4-dibromooestrone was crystallised from dichloromethane–methanol to a constant isotope ratio. In reasonable agreement with literature [7,8] it had m.p. 228–230°C, $[\alpha]_D$ + 121°, λ_{max} 292 nm (ϵ 2900) with shoulder at 285 nm and λ_{max} 313 nm (ϵ 5500) in 0.1 M ethanolic NaOH, ν_{max} 1710 cm^{-1} (17-one) and 875 cm^{-1} (lone arom. H).

Bromination with 1 mol. equiv. reagent followed by elution of the product with chloroform from a column of alumina (Camag, neutral, act. I) and then by successive crystallisation from dichloromethane–acetone and dichloromethane–methanol gave [4-^{14}C]-[2,6α,7α-^{3}H]4-bromooestrone of a constant isotope ratio. After the first crystallisation the m.p. was 288–292°C, after the second crystallisation it was 266–268°C; literature records m.p. values of 264–265°C [7], 281–283°C [9] and 280–281°C [10]. The product had $[\alpha]_D$ + 122° (in dioxan; cited lit.: + 134–147°, in chloroform), λ_{max} 282 nm (ϵ 2550) with shoulder at 291 nm and λ_{max} 304 nm (ϵ 4100) in 0.1 M ethanolic NaOH, ν_{max} 1718 cm^{-1} (17-one) and 820 cm^{-1} (2 adjacent H atoms).

2.6. *HRP-catalysed oxidation of oestrone*

The reaction was performed as with oestradiol [4] except that the concentration of oestrone was lower (67 mg/l buffer). After precipitation from dioxan–ether the pure product was obtained in 10% yield. It had m.p. 280–286°C, $[\alpha]_D$ + 135°C, inflection at 275 nm (E_1^1 122).

Found: C, 77.5; H, 8.1%.

Calc. $(C_{18}H_{20}O_2)_n \cdot \frac{n}{2}(H_2O)$: C, 77.95; H, 7.6%.

Calc. $(C_{18}H_{20}O_{2.5})_n$: C, 78.2; H, 7.3%.

Identity with the ferricyanide oxidation product was confirmed by mixed m.p. and by comparison of the i.r. spectra of the two samples.

2.7. *Ferricyanide oxidation of oestradiol 17-monoacetate*

Oestradiol 17-monoacetate (400 mg) was treated with potassium ferricyanide in the usual manner. Out of 4 mol. equiv. reagent available 2.2 mol. equiv. were consumed. The product (267 mg) was eluted from a column of alumina with benzene–chloroform (1:1, v/v). Incipient precipitation from ethanol gave a dull-yellow material (140 mg) of m.p. (dec.) 256–261°C, $[\alpha]_D$ + 30°, inflection at 275 nm (E_1^1 125), ν_{max} 1735 cm^{-1} (acetate C–O) and 1250 cm^{-1} (acetate C–O), τ (220 MHz, in perdeuteroacetone) 2.95 and 3.4 (arom. H), 8.0 (CH_3COO) and 9.18 (18-CH_3). Mol wt by vapour pressure osmometry: 2220 in chloroform, 1267 in dioxan; by Rast's method: 1307.

Found: C, 74.7; H, 7.7%.

Calc. $(C_{20}H_{24}O_3)_n \cdot \frac{n}{2}(H_2O)$: C, 74.7; H, 7.8%.

Calc. $(C_{20}H_{24}O_{2.5})_n$: C, 75.0; H, 7.55%.

3. Results and discussion

The lactoperoxidase-catalysed conversion of oestradiol into the product obtained by the action of ferricyanide and of HRP–H_2O_2 [4] strengthens the case for examining the possibility that the same conversion is brought about by the uterine peroxidase [1,2] and by the peroxidase from an induced mammary tumour with properties similar to those of lactoperoxidase [3]. The corresponding transformations of oestradiol 17-monoacetate and of oestrone were brought about with ferricyanide and that of the latter compound also with HRP–H_2O_2. All three products are dull-yellow, amorphous, hygroscopic and non-volatile. Their elemental compositions are consistent with those of oligomers or polymers containing one additional oxygen atom or one molecule of water for every two monomer sub-units. Their u.v. spectra are essentially identical. Their. i.r. spectra are similar except for differences due to different substituents at C-17.

Vapour pressure osmometry of the three products in dioxan gave molecular weights of tetramers whilst those of octamers were determined for the oestradiol oxidation product in ethanol and for the other two products in chloroform. By Rast's method, the last two products had molecular weights of tetramers. These findings pointed to the formation of tetramers which in solvents less strongly hydrogen-binding than dioxan associate to octamers. However, the nuclear magnetic resonance spectra of the oxidation products of oestrone and oestradiol 17-monoacetate exhibited extremely broad bands (height over width at half-peak height approx. 20:1 for the C-18 methyl protons and 1:1 for the aromatic protons) strongly suggesting polymeric character. The NMR spectra also showed the presence of an average of 1.5–2 aromatic protons for each monomer sub-unit of the polymer.

Oxidation of [4-^{14}C] [2,4,6α,7α-^{3}H]oestrone with 4 and with 1 mol. equiv. ferricyanide proceeded with the loss of 29% and of 29.6% tritium, respectively. In the latter experiment the unreacted oestrone was recovered with an unchanged isotope ratio and hence the operation of a kinetic isotope effect need not be considered. Displacement of tritium from position 6 and 7 was ruled out by oxidation of [4-^{14}C][6α,7α-^{3}H]oestrone without loss of tritium. Bromination of [4-^{14}C] [2,4,6α,7α-^{3}H]oestrone gave the 2,4-dibromo derivative with the loss of 49% tritium and partial bromination gave the 4-bromo derivative with the loss of 23% tritium. The total amount of tritium at positions 2 and 4 found in a sample of [2,4,6α,7α-^{3}H]-oestrone by tritium NMR spectrometry was 49% and after the compound's reduction to oestradiol 32% were found at C-2 and 15% at C-4 [11]. As tritium in the starting material was more abundant at C-2 than at C-4, the loss of 29% tritium on oxidation definitely implicates position 2 as a site of reaction. It is therefore of interest to note that HRP catalyses the iodination of oestrogens at position 2 [12]. Oxidation of [4-^{14}C] [6α,9-^{3}H]oestrone without loss of tritium rules out reaction at position 9 unless fission of the C_9–C_{10} bond occurred, as in the HRP-catalysed formation of S(3,17β-di-hydroxy-9,10-secooestra-1,3,5(10)-trien-9-on-10-yl)-glutathione from oestradiol and glutathione [13].

The oestrone polymer was not acetylated by acetic anhydride in pyridine nor was it methylated by diazomethane. Its phenolic character – as that of the oestradiol polymer [4] – was indicated by an alkali-induced bathochromic shift of its u.v. absorption band. Its i.r. spectrum in chloroform showed a band at 3530 cm^{-1} and a weaker one at 3450 cm^{-1} whose intensities were unaffected by concentration. The former band is attributed to intramolecular OH···π-electrons association and the latter to OH···O association [14–16]. In dioxan, the OH stretching band was very broad with a maximum at 3350 cm^{-1} and shoulders at 3420 cm^{-1} and 3500 cm^{-1}. Similar shifts in dioxan were reported for 2,2′-dihydroxybiphenyl and its monomethyl ether [17].

It is concluded that:

1. Oestrogens undergo the same transformations by the action of peroxidases as by that of potassium ferricyanide.
2. The transformation products are polymers. The apparent molecular weights determined by osmometry and by Rast's method remain unexplained.
3. The polymers contain an unknown number of unreactive phenolic groups.
4. Position 2 is a site of reaction but participation of position 4 cannot be ruled out on present evidence.
5. α-Substitution at C-6 is ruled out and β-substitution at the same site seems unlikely on steric grounds.
6. Reaction at C-9 is ruled out unless it involves fission of the C_9–C_{10} bond.

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