BBA 63369

Mechanism of hydrolysis of estradiol 3-phosphate by placental acid phosphatase III

We have recently described the isolation of three enzymes with acid phosphatase activity from human placenta[1], one of which was characterized by a marked specificity for certain steroid phosphates, notably 17 β-estradiol 3-phosphate[2]. In view of the unique properties of this enzyme it was of interest to determine which bond is broken in the hydrolytic reaction, in accordance with the idea that the break comes nearest that part of the molecule of greatest specificity to the enzyme[3]. The identification of the bond affected was accomplished by carrying out the reaction in water enriched with ^{18}O and determining which reaction product incorporates the isotope: thus, P–O fission would result in incorporation of ^{18}O in orthophosphate, while C–O fission would result in ^{18}O-labeled estradiol.

Water containing 10.0 atom % excess ^{18}O and 0.2 atom % excess ^{17}O was obtained from Oak Ridge National Laboratory and distilled in an all-glass apparatus before use. Synthetic estradiol 3-phosphoric acid was prepared as described previously[2] and dissolved in 50 mM sodium citrate buffer to give a saturated solution at room temperature and pH 6.0 which contained 7.5 mM of the phosphate. A 1.0-ml aliquot of this solution was lyophilized and reconstituted with 1.2 ml of the isotopic water. Human placental acid phosphatase III was partially purified by Sephadex gel filtration and ion-exchange chromatography to a specific activity of 2 I.U./mg protein at 37°. To start the reaction, 50 μl of this enzyme solution containing 0.1 unit of activity was added to the above 1.2 ml of reaction mixture. Hydrolysis of the phosphate group proceeded rapidly at room temperature resulting in the precipitation of sparingly soluble 17 β-estradiol, which was centrifuged off and recrystallized from hot aqueous ethanol. A few crystals of this material were analyzed with a LKB mass spectrometer, Type 9000 (Stockholm), and the mass spectrum compared to that obtained with a recrystallized sample of 17 β-estradiol purchased from Steraloids (Pawling, N.Y.). The samples were introduced through the direct inlet system and run at 70 eV, ion source temperature, 250°, ionizing current, 65 μA, accelerating voltage, 3.5 kV. The sample temperature at peak ion production was 100–120°. Perflurokerosene (Pierce Chemical Co., Rockford, Ill.) was used as a mass marker.

Mass spectra of estradiol, and the reaction product from the enzymic hydrolysis of estradiol 3-phosphate in ^{18}O-enriched water are presented in Fig. 1 for comparison. The prominence of the molecular ion at *m/e* 272, as well as the typical steroid fragmentation product at *m/e* 213, resulting from loss of the 17-hydroxyl group *plus* 42 mass units[4], are seen in both spectra. With regard to the major peaks, the two spectra are virtually identical, leaving little doubt as to the identity of the enzymic reaction product. An analysis of these spectra in the vicinity of the molecular ion is presented in Table I. The intensities of the $(M + 1)^+$ and $(M + 2)^+$ peaks are expressed as percentages of the molecular ion (M^+) peak. The amounts of $(M + 1)^+$ and $(M + 2)^+$ produced from both compounds are seen to agree well with the amounts of these molecular species predicted from the natural abundance of the stable isotopes of carbon, hydrogen and oxygen[5]. The absence of any increase in the relative intensity of the $(M + 2)^+$ peak over the theoretical value of 2.37 is taken as evidence that no

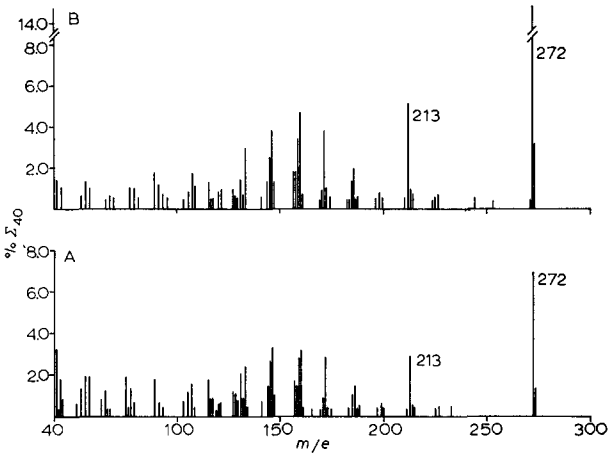

Fig. 1. Mass spectra of (A) a commercial recrystallized sample of 17β-estradiol, and (B) the isolated reaction product from the hydrolysis of 17β-estradiol 3-phosphate by placental phosphatase III, carried out in 10% ^{18}O-enriched water. The relative intensity of each ion fragment is expressed in % of $\Sigma 40$, which is the sum of the intensities of all peaks from mass 40 to mass 274. Values of % $\Sigma 40$ less than 0.30 are not given.

extra ^{18}O was incorporated into the estradiol by cleavage of the phosphate group in $H_2{}^{18}O$. It follows therefore that the hydrolysis proceeds exclusively through fission of the P–O bond.

Previous studies have revealed that the P–O bond is attacked by four other phosphate-transferring enzymes: acid and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2 and 3.1.3.1), phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) and ribonuclease (polyribonucleotide 2-oligonucleotidotransferase, EC 2.7.7.16)[6]. However, all of these enzymes have wide substrate specificities, in contrast to the recently described placental phosphatase (III) which hydrolyses only a limited number of monophosphate esters. Other phos-

TABLE I

DISTRIBUTION OF STABLE ISOTOPES IN 17β-ESTRADIOL: ANALYSIS FOR EXCESS ^{18}O

The theoretical percentages of molecules of estradiol having molecular weights of 272, 273, and 274 was calculated from the natural abundance of the isotopes: ^{13}C (1.13%), ^{2}H (0.02%), ^{17}O (0.037%) and ^{18}O (0.204%). The actual distribution of these molecular species was determined from the relative intensities of the corresponding ions in the mass spectrum of the sample compounds. M^+ represents the molecular ion or "parent peak" from estradiol.

Ion peak	*m/e*	*Relative ion intensity*		
		Estradiol reference standard	*Sample from hydrolysis of estradiol phosphate in* $H_2{}^{18}O$	*Theoretical*
M^+	272	(100.0)	(100.0)	(100.0)
$(M + 1)^+$	273	19.0	20.6	20.8
$(M + 2)^+$	274	2.1	2.3	2.37

phatases with a narrow range of substrate specificity, such as hexosediphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) have presumably not been systematically investigated as to identification of the bond cleaved. Despite the results of the present work it is probably premature to assume, as some have[6,7], that cleavage of the P–O bond may be a general feature of phosphatase action. Further study of the mechanism of action of some of the socalled "substrate-specific" phosphatases is indicated here.

This work was supported in part by the Ford Foundation and the Population Council Grant No. M-68.84. The author is indebted to Mr. C. T. Wetter of the Chemistry Department of Vanderbilt University for technical assistance in determining the mass spectra.

Department of Obstetrics and Gynecology,
Vanderbilt University School of Medicine,
Nashville, Tenn. 37203 (U.S.A.)

DAVID L. DIPIETRO

1 D. L. DIPIETRO AND F. S. ZENGERLE, *J. Biol. Chem.*, 242 (1967) 3391.
2 D. L. DIPIETRO, *J. Biol. Chem.*, 243 (1968) 1303.
3 D. E. KOSHLAND AND S. S. STEIN, *J. Biol. Chem.*, 208 (1954) 139.
4 K. BIEMANN, *Mass Spectrometry*, McGraw-Hill, New York, 1962, p. 339.
5 R. I. REED, *Applications of Mass Spectrometry to Organic Chemistry*, Academic Press, New York, 1966, p. 18.
6 E. C. WEBB, *Enzymes*, Academic Press, New York, 1964, p. 281.
7 R. K. MORTON, in M. FLORKIN AND E. H. STOTZ, *Comprehensive Biochemistry*, Vol. 16, Elsevier, Amsterdam, 1965, p. 59.

Received October 21st, 1968