Comparison of Radioactive Peptides Obtained from Specifically Labelled Human Renal and Placental Alkaline Phosphatases

By Katrine B. WHITAKER and Donald W. MOSS

Royal Postgraduate Medical School (University of London), Hammersmith Hospital,
London W12 0HS, U.K.

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Peptides with different chromatographic and electrophoretic properties were obtained from human placental and renal alkaline phosphatases by tryptic digestion of the enzymes labelled with radioactive orthophosphate at their active centres. These results provide structural evidence for the distinct genetic origins of the two isoenzymes that had previously been inferred from their different properties and from the observed phenotypic variation of placental phosphatase.

Alkaline phosphatases (orthophosphoric monoester phosphohydrolases, EC 3.1.3.1) from various human tissues have been shown to differ by several criteria, the most marked differences existing between placental and non-placental phosphatases (Moss, 1970). The placental isoenzyme has been highly purified and its amino acid composition determined (Harkness, 1968; Ghosh & Fishman, 1968; Sussman & Gottlieb, 1969). Other human alkaline phosphatases have also been purified extensively (e.g. Eaton & Moss, 1968; Trépanier *et al.*, 1976; Komoda & Sakagishi, 1976; Latner & Hodson, 1976; Gerbitz *et al.*, 1977). However, structural analysis of these non-placental enzyme forms is less advanced, although differences in two-dimensional peptide 'maps' between human hepatic and placental alkaline phosphatases have been reported (Badger & Sussman, 1976). Alkaline phosphatases from various sources have been shown to incorporate radioactively labelled orthophosphate at acid pH, with formation of a phosphoserine residue (Engström, 1961; Schwartz & Lipmann, 1961; Barman & Gutfreund, 1966; Moss *et al.*, 1968), and this property has been used to study peptides derived from the active centre of the enzyme, identified by the presence of the label after partial hydrolysis of labelled phosphatases (Schwartz *et al.*, 1963; Milstein, 1964; Engström, 1964; Whitaker *et al.*, 1976). The method does not demand complete purity of the phosphatase under study, provided that no other protein capable of incorporating P_i is present. We have previously reported some properties and the probable amino acid composition of an active-site peptide obtained by tryptic digestion of radioactively labelled human placental alkaline phosphatase (Whitaker *et al.*, 1976). The present paper shows differences between the placental peptide and peptides obtained by similar means from the alkaline phosphatase of human kidney.

Experimental

Purification of alkaline phosphatase

Human placental alkaline phosphatase was purified to a specific activity of 550 units (μmol of *p*-nitrophenol liberated/min)/mg of protein (Whitaker & Moss, 1976). Human kidney was homogenized with water (1 ml/g of tissue), extracted with butan-1-ol (1.5 ml/g of tissue) and alkaline phosphatase was recovered by fractional precipitation with acetone between 45% (v/v) and 60% (v/v). The enzyme was eluted from a column (70 cm $\times$ 2.5 cm) of TEAE-(triethylaminoethyl)-cellulose (Sigma Chemical Co., St. Louis, MO, U.S.A.) in Tris buffer (0.01 M; adjusted to pH 7.7 with HCl at 25°C) containing zinc acetate (0.1 mM) with a linear gradient of increasing NaCl concentration (0–0.2 M). Enzyme eluted at above 0.07 M-Cl⁻ corresponded to the small proportion of intestinal-like alkaline phosphatase present in kidney (Boyer, 1963) and was discarded. The protein (0.31 g) with phosphatase activity eluted at Cl⁻ concentrations between 0.01 and 0.07 M was incubated for 24 h at 37°C with about 5 units of neuraminidase (type V, from *Clostridium perfringens*; Sigma Chemical Co.) in the same Tris buffer to minimize heterogeneity with respect to charge. The neuraminidase-treated enzyme was again separated on TEAE-cellulose as described above, when most of the phosphatase was eluted in a single peak at zero Cl⁻ concentration. This peak was applied to a column (70 cm $\times$ 2.6 cm) of Sephadex G-200 (40–120 μm particle size; Pharmacia, Uppsala, Sweden) in Tris buffer (0.01 M; adjusted to pH 7.7 with HCl at 25°C) containing NaCl (0.1 M). Except where indicated all operations were performed at 4°C. The resulting kidney phosphatase preparation was purified 600-fold overall to a specific activity of 180 units/mg of protein.

Alkaline phosphatase activity was measured with *p*-nitrophenyl phosphate as substrate at 37°C as recommended by the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1974). Protein was determined either by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, or by measurement of absorbance at 280 nm, assuming that $1.4 \frac{A_{280}^{1\text{cm}}}{\text{unit}}$ corresponds to a protein concentration of 1 mg/ml.

Preparation and analysis of labelled peptides

The procedure for labelling alkaline phosphatase was essentially as previously described (Whitaker *et al.*, 1976). Human placental or human kidney alkaline phosphatase preparations (0.1 ml) were added to carrier-free [^{32}P]P_i (2 mCi in 0.2 ml; The Radiochemical Centre, Amersham, Bucks., U.K.), sodium acetate buffer (0.4 ml; 50 mM; pH 5) and Na₂HPO₄ at a final concentration of P_i of 1 mM for placental phosphatase and 0.5 mM for the kidney enzyme. After 5 min at 0°C, protein was precipitated with 0.1 ml of HClO₄ (4 M) and washed several times with HClO₄ (0.5 M) to remove unbound P_i. Radioactivity was measured by liquid-scintillation counting of 10–200 μl samples with 10 ml of Insta-gel (Packard Instrument Co., Downers Grove, IL, U.S.A.) with internal standards. Both enzyme preparations lost activity during storage between purification and labelling, so that the 400 μg of protein present in 0.1 ml of the solutions corresponded to 36 units for placental phosphatase and 17 units for the kidney enzyme at the time of labelling. The amounts of bound P_i in the placental and kidney alkaline phosphatase precipitates were 580 pmol and 3 pmol respectively.

Tryptic digestion was performed for 24 h at 37°C in solutions containing 1 mg of protein/ml and 50 μg of trypsin (type XI, diphenylcarbamoyl chloride-treated, from bovine pancreas; Sigma Chemical Co.)/ml in (NH₄)₂CO₃ (0.5 M). In some experiments, tryptic peptides were completely hydrolysed by incubating them for 72 h at 37°C with aminopeptidase (aminoacyl-oligopeptide hydrolase, from pig kidney; Sigma Chemical Co.) at a concentration of about 0.8 mg/ml in Tris buffer (0.01 M; adjusted to pH 7.0 with HCl at 25°C).

Gel filtration of peptides was carried out on a column (28 cm $\times$ 0.9 cm) of Bio-Gel P-4 (200–400 mesh size; Bio-Rad Laboratories, Richmond, CA, U.S.A.) with a solution of formic acid (0.1 M) and propan-2-ol (10%, v/v) as eluent. The column was calibrated with the peptides [leucine]enkephalin, [arginine]vasopressin, luliberin (luteinizing-hormone-releasing factor), somatostatin, a fragment of human calcitonin (amino acids 17–32) and human calcitonin M.

Two-dimensional peptide 'maps' were obtained by electrophoresis followed by chromatography on

thin-layer cellulose plates (20 cm $\times$ 20 cm; Merck, Darmstadt, Germany). Electrophoresis was carried out in formic acid/acetic acid/water (1:4:45, by vol.; pH 2) at 1000 V for 30 min and chromatography for 4 h with butan-1-ol/pyridine/acetic acid/water (21:12:2:15, by vol.) as solvent. The labelled peptides were located by radioautography with Kodirex Auto-process film (Kodak, Hemel Hempstead, Herts., U.K.). Phosphoserine was stained by spraying the plate with ninhydrin [0.1% (w/v) in 2,4,6-trimethylpyridine/acetic acid/ethanol (2:15:50, by vol.)] and heating to 100°C to develop the colour.

Results and Discussion

The specificity of labelling of kidney alkaline phosphatase with [^{32}P]P_i was confirmed by the parallel decline in ability to incorporate the label and in catalytic activity after incubation of the enzyme solution at 56°C. Values for radioactivity incorporated and residual catalytic activity (in parentheses) after 5, 10 and 20 min incubation were 36.4 (34.0), 15.5 (17.2) and 3.8 (6.8) respectively. Both properties are expressed as percentages of values for unheated controls. Further evidence of the specificity of labelling was obtained by digestion with aminopeptidase of labelled tryptic peptides from renal phosphatase recovered after electrophoresis. The major radioactive product obtained was electrophoretically identical with phosphoserine. The same result was obtained with placental phosphatase. Incorporation of label into placental phosphatase was apparently 90-fold greater than that into the kidney isoenzyme. This may imply a higher catalytic-centre activity on the part of the latter isoenzyme. Some evidence for this can be found in earlier labelling experiments, in which greater catalytic-centre activities were observed for non-placental than for placental alkaline phosphatases (Moss *et al.*, 1968; Whitaker & Moss, 1976). However, kidney phosphatase is less stable than placental phosphatase, so that more denaturation of the kidney isoenzyme under the acid conditions of labelling might also contribute to its lower incorporation of label.

After tryptic digestion of the labelled kidney phosphatase, two closely spaced radioactive spots, together with a third spot corresponding to free [^{32}P]P_i, were present on radioautograms of two-dimensional electrophoretic and chromatographic separations (Fig. 1). Similar analysis of tryptic digests of labelled placental phosphatase showed a single major radioactive peptide with traces of other products (Fig. 1), as previously observed (Whitaker *et al.*, 1976). However, the radioactive-peptide 'maps' obtained for the kidney phosphatase and for the placental enzyme had no points of similarity between them (with the exception of unbound label) (Fig. 1). Gel filtration on Bio-Gel P-4 of the major

Fig. 1. Tracings of radioautograms of two-dimensional separations of tryptic digests of human alkaline phosphatases labelled with $[^{32}\text{P}]\text{P}_i$.

(a) Digest of kidney phosphatase (215 d.p.s. applied; exposure 18 h); (b) digest of placental phosphatase (1150 d.p.s. applied; exposure 3.5 h); (c) mixture of digests of kidney and placental phosphatases (455 d.p.s. applied; exposure 18 h). Electrophoresis was followed by chromatography on thin-layer cellulose plates (see the text for details). Origins are arrowed. P represents unbound $[^{32}\text{P}]\text{P}_i$; R, radioactive peptides submitted to further analysis. Approximate relative intensities of spots are shown by filled, continuous or broken outlines.

radioactive peptides recovered after electrophoresis showed that the peptides from kidney phosphatase were different in size from the peptide from placental phosphatase (Fig. 2), with an apparent molecular weight of 1800 for the kidney peptides compared with 2300 for the peptide from the placental enzyme (Whitaker *et al.*, 1976).

The main advantage of a method such as the one employed here lies in its specificity: this removes the need to assume complete purity of the isoenzyme preparations, although some purification is necessary to remove other proteins that may take up the label and to ensure an adequate specific radioactivity of labelled products. However, the ability of the method to discriminate between two isoenzymes is potentially limited by the probability that the primary structure in the region of the active centre has been conserved to a large extent during evolution, so that differences in structure will not be apparent in those parts of the polypeptide chains comprising active-centre peptides. A comparison of the amino acid compositions of active-site peptides from *Escherichia coli* alkaline phosphatase (Schwartz *et al.*, 1963) and the human placental isoenzyme (Whitaker *et al.*, 1976) shows that considerable possibilities of such conservation do exist. Furthermore, Zwaig & Milstein (1964) found nearly identical patterns of radioactive peptides after partial acid hydrolysis of ^{32}P -labelled alkaline phosphatases from two bacterial species. However, the present results show that differences in

Fig. 2. Chromatography on Bio-Gel P-4 of radioactive peptides from alkaline phosphatases

The peptides were partially purified by electrophoresis from tryptic digests of human kidney (●) and placental (○) alkaline phosphatases labelled with $[^{32}\text{P}]\text{P}_i$. The void volume is arrowed.

primary structure between two organ-specific human alkaline phosphatases can be revealed by the comparison of specifically labelled peptides. Since the kidney phosphatase peptide is smaller than that from placental phosphatase, the former enzyme presumably contains one or more lysine or arginine residues in the neighbourhood of the reactive serine residues,

forming trypsin-sensitive bonds that are replaced by different residues in placental alkaline phosphatase. It is noteworthy that the known sequence close to the reactive serine residues of *E. coli* alkaline phosphatase contains lysine as the seventh residue towards the *N*-terminus of the chain (Schwartz *et al.*, 1963). This residue may have been conserved in human renal alkaline phosphatase but replaced in the placental isoenzyme.

The existence of an independent gene locus governing the structure of human placental alkaline phosphatase has been deduced from the marked phenotypic variation of this isoenzyme (Donald & Robson, 1974), which is not shared by alkaline phosphatases from other human tissues. It remains to be seen whether the method of radioactive peptide 'mapping' is able to distinguish between the closely similar alkaline phosphatases from non-placental tissues such as liver, bone and kidney, and therefore to allow inferences about their genetic origins to be drawn.

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