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Effect of the phosphonic analogue of tyrosine on tyrosine iodination

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Summary. Depositing of DL-1-amino-2-(*p*-hydroxyphenyl)-ethylphosphonic acid (Tyr-P) on the chicken embryo induced a dose dependent decrease of the iodine uptake by the embryonic thyroid. Tyr-P interfered on iodination of tyrosine when tested with hog thyroid peroxidase (TPO) and with bovine lactoperoxidase (LPO); the analogue was recognized by the two enzymes but its affinity for TPO and LPO was respectively 3 and 7 fold higher compared with that of the natural substrate, suggesting that Tyr-P may act as an iodine trap.

Keywords: Amino acids – Bovine lactoperoxidase – Thyroid peroxidase – Tyrosine derivatives – Phosphonic analogue of tyrosine.

Introduction

The biosynthesis of thyroid hormones, besides its well known inhibition by iodine, is also inhibited by a number of antithyroidic agents which are able to correct the disturbances of regulatory mechanisms of the biosynthesis and therefore may show a therapeutic interest. These agents can be classified in two main groups, according to their site of action. We first have to consider the inhibitors of iodide transport: (i) monovalent anions especially thiocyanate (Michot et al., 1980), (ii) ouabain, acting on Na^+/K^+ dependent ATPase, (iii) azide, at the level of aerobic metabolism, (iv) 2,4-dinitrophenol, an uncoupler of phosphorylative oxidations. The second group includes several molecules interacting with thyroid peroxidase (TPO) and with iodotyrosine deiodinase. Among them, tyrosine analogues present a potential interest: α-methyl-tyrosine (AMT), though a substrate and an inhibitor of TPO, only shows a limited antithyroidic effect; α-methyl-dihydroxyphenylalanine (AMD) is a non competitive inhibitor of the enzyme (De Groot and Jaksina, 1969); the *meta*-substituted derivatives of tyrosine, particularly dibromotyrosine, are competitive inhibitors of iodotyrosine deiodination.

Phosphonic analogues of aminoacids (in which the Cα-COOH group is replaced by a Cα-PO_3H_2 group) present original properties regarding their interactions with natural aminoacid metabolism (Kafarski and Mastalerz, 1984). The phosphonic analogue of tyrosine has been described to be a useful investigation tool in the field of tyrosine metabolism (Iron et al., 1981). So, Tyr-P acts as a good inhibitor on decarboxylation of Tyr and DOPA (Iron et al., 1986). A strong inhibitory effect has been also demonstrated on tyrosine transamination (Iron et al., 1993). Moreover, Tyr-P is known to interfere on thyroid metabolism: it can be chemically iodinated and converted to its mono and diiodo derivatives (Lacoste et al., 1967a). The phosphonic analogue of T_4 possesses a thyromimetic action on the tadpole (Lacoste et al., 1967b) and on the chicken embryo (Lacoste et al., 1968). In this paper, we study the *in vivo* effect of Tyr-P on chicken embryo thyroid metabolism and its behaviour *in vitro* on the iodination reaction catalyzed by bovine lactoperoxidase (LPO) and hog thyroid peroxidase (TPO).

Materials and methods

Chemicals

Lactoperoxidase (LPO) was purchased from Boehringer-Mannheim (Germany); hog thyroid peroxidase (TPO) was prepared according to Coval and Taurog (1967). L-Tyr was from Fluka, Switzerland. The Tyr analogues came from Calbiochem, USA: DL-Tyr-P, D-Tyr, 3-iodo-L-Tyr (MIT), 3,5 diiodo-L-Tyr, 2 H_2O (DIT); from Aldrich, USA: α-methyl-DL-Tyr(AMT); from Cyclo, USA: *m*-DL-Tyr, *p*-hydroxyphenylpyruvate (*p*HPP), di-iodothyronine (T_2); from Roche, France: thyronine, tri-iodo-thyronine (T_3), thyroxine (T_4). MIT-P and DIT-P were synthesized in our laboratory (Lacoste et al., 1967). ^{131}I and U-^{14}C-Tyr were from CEA, France.

Assays on chicken embryo

Various amounts of Tyr or Tyr-P at pH 8 and a trace dose of ^{131}I were applied upon the vitellus of fertilized eggs. After 14 or 18 days of incubation, embryos were sacrificed and weighed. The two lobes of the thyroid gland were separated and their radioactivity measured using a Geiger-Müller counter. Each experiment was paralleled by a control assay in which NaCl – in the same conditions of pH and ionic strength – replaced the tested molecule.

For each series, the mortality rate, the average weight of embryos and the average radioactivity (cpm per gram of embryo) were determined. Tests series at 14 days of incubation included 16, 16, 18, 16 and 14 eggs for Tyr-P doses of 1, 5, 10, 20 and 40 μmoles respectively; the number of eggs was 12, 13, 16, 14 and 11 in the control series. Approximately the same number of eggs constituted the two series at 18 days of incubation.

Iodination kinetics by triiodide ion evaluation

The iodination rate of the substrates was determined by measuring triiodide (I_3^-) ion formation (Morrisson and Baysse, 1967). After an incubation time of 30 sec at room temperature, the specific absorbance of I_3^- was plotted during 1 min at 353 nm; the reaction rate was expressed as $\Delta A(I_3^-)$/min. For LPO-catalyzed iodination, the reaction mixtures included in a final volume of 2.6 ml: phosphate buffer pH 7.4, 50 mM; 0.1 mM KI; 0.1 mM H_2O_2; substrate: various concentrations; the reaction was initiated by addition of 0.1 unit of LPO giving a final enzyme concentration of 7.8 nM. For TPO-catalyzed iodination, the experimental conditions were: final volume, 2.75 ml; phosphate buffer pH 7.4, 50 mM; 10 mM KI; TPO preparation 100 μl, substrate: various concentrations. The addition of H_2O_2 (final concentration: 0.1 mM) started the reaction.

Identification of iodinated products

The identification of tyrosine and thyronine iodinated derivatives (MIT, DIT, T_2, T_3 T_4). was carried out using similar mixtures as described above for iodination by LPO; the substrate concentration was 1 mM. The reaction was developed at 37°C for 5 min and stopped by 3 M trichloroacetic acid. An aliquot of the supernatant was then submitted to a paper electrochromatographic separation: the *n*-butanol/acetic acid/water (12 : 3 : 5, vol/vol) chromatography (18 h) was followed by electrophoresis performed at 2000 V for 90 min (methanol/acetic acid/water, 78 : 25 : 897, vol/vol; pH: 1.85). The spots were revealed either by ninhydrin or by a ceric sulfate reagent specific for iodinated products (Pitt-Rivers and Schwartz, 1969). The identification was done by comparing the R_f values of the spots with the Rf values of control molecules separated in the same conditions. A simpler protocol, limited to the chromatographic separation, was adopted to detect a deiodinase activity of TPO with 2 mM MIT or 2 mM DIT as substrate.

Quantitation of inhibitory effects

For the quantitative study of the action of Tyr-P on tyrosine iodination, ^{14}C-Tyr (473 mCi/mmol) was used in a protocol similar to those described above for LPO- and TPO-catalyzed iodination; the incubation time was limited to 5 min; L-Tyr was used either alone (at a final concentration of 0.18 mM) or mixed with Tyr-P 1.8 mM ([Tyr-P]/[Tyr] = 10). The radioactivity of the spots was measured after the chromatographic separation. For the two enzymes experimented, five assays were performed and the mean values determined.

Statistical analysis

Fisher's test was used for comparison of variances and Student's test for comparison of means.

Results

In vivo experiments

A important mortality of the chicken embryos was observed when larger doses of Tyr-P were deposited upon the vitellus: 20 and 40 μmoles led only to a 62 and 7 per cent survival, respectively, at the 14th day of incubation. However, no significant differences in weight could be noted for the survivors. As sodium chloride, assayed at the same concentrations of 20 and 40 μmoles, also induced some mortality (21 and 27 per cent), a non specific action of the ionic strength on mortality must be considered.

Consequently, our experiments were limited to low concentrations of the tyrosine analogue, with an upper limit of 10 μmoles. We noted a significant decrease in the test series versus the control series ($p < 0.05$) up to 10 μmoles of Tyr-P. (Fig. 1). Beyond this dose, the increase of the ionic strength cancelled the specific effect of Tyr-P. After an incubation period of 18 days, the effect was similar but more important than after 14 days.

In vitro experiments

When L-Tyr and DL-Tyr-P were iodinated by LPO and TPO, they showed a Michaelian kinetics; K_M and V_{max} are given in Table 1. These values agree with

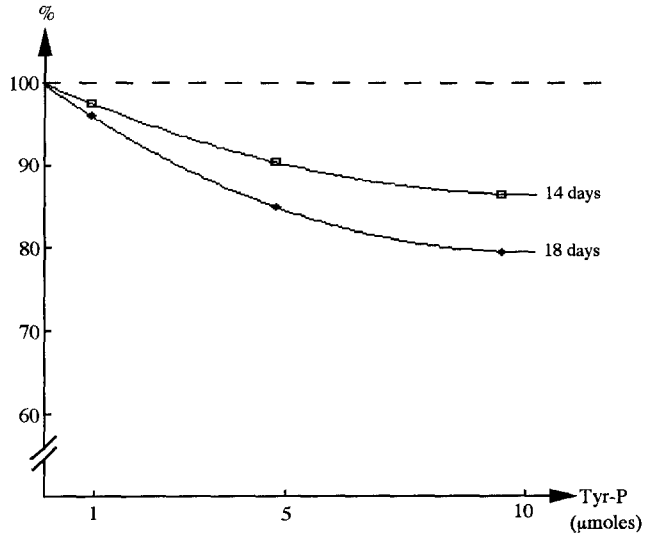

Fig. 1. Iodine intake by chicken embryo thyroid after an incubation of 14 days and of 18 days. Results are expressed in percentage of thyroid radioactivity reported to the radioactivity of the control series

Table 1. Iodination by bovine lactoperoxidase (LPO) and hog thyroid peroxidase (TPO). K_M and V_{max} values for L-Tyr, DL-Tyr P and analogues. V_{max}: arbitrary units

	Bovine lactoperoxidase		Hog thyroid peroxidase	
	K_M (μM)	V_{max}	K_M (μM)	V_{max}
L-Tyr	16	1	70	0.8
DL-Tyr-P	110	0.9	230	2.8
D-Tyr	20	0.9	100	0.98
DL-*m*-Tyr	125	1.43	140	0.94
DL-α-Methyl-Tyr	27	0.83	1000	3.33
p-Hydroxyphenylpyruvate	31	1.81	210	2.17

the K_M values given in the literature for L-Tyr: 13 μM for LPO (Morrison and Baysse, 1970) and 65 μM for TPO (Pommier et al., 1972).

In order to test the specificity of both LPO and TPO iodinating enzymes, four additional Tyr analogues compounds were used to evaluate the importance of stereoisomerism (D-Tyr), of the position of the phenol hydroxyl (DL-*meta* Tyr), of the replacement of the α-hydrogen (DL-α methyl Tyr) and of the removal of the amine function (*p*-hydroxyphenylpyruvic acid). The kinetics were of the Michaelian type; the corresponding constants are given in Table 1.

LPO iodination allowed the electrochromatographic detection of thyroxine (T_4) and of its precursors, MIT, DIT and T_2; the spots were identified by a ninhydrin spray, by the ceric sulfate reagent and by comparison with control

substances. The DIT spot was always more intense than the spot of the monoiododerivative. No T_3 could be detected.

As the V_{max} value of TPO towards Tyr-P appeared to be three fold higher than that of LPO, experiments were carried out with the hog enzyme: Tyr-P was partially converted into its mono and diiodo derivatives, MIT-P and DIT-P, which were identified using the control substances (Cassaigne, 1968).

To study the influence of Tyr-P on the enzymatic iodination of Tyr, the enzymatic iodination assays were performed as mentioned above (L-Tyr, 1.8 mM; DL-Tyr-P, 18 mM) in the presence of a tracer dose of ^{14}C-L-Tyr; iodinated products were identified after their chromatographic separation; the radioactivity was measured on the isolated ninhydrin-positive spots. Tyr-P did not modify the distribution of the radioactivity when LPO was used for the enzymatic iodination (Fig. 2A). The results obtained with TPO were quite different (Fig. 2B): a larger amount of DIT (+16%) was observed, paralleling the noticeable decrease of L-Tyr (−18%); MIT, the intermediary iodinated derivative was non significantly modified. Thyroxine production was severely reduced (−44%).

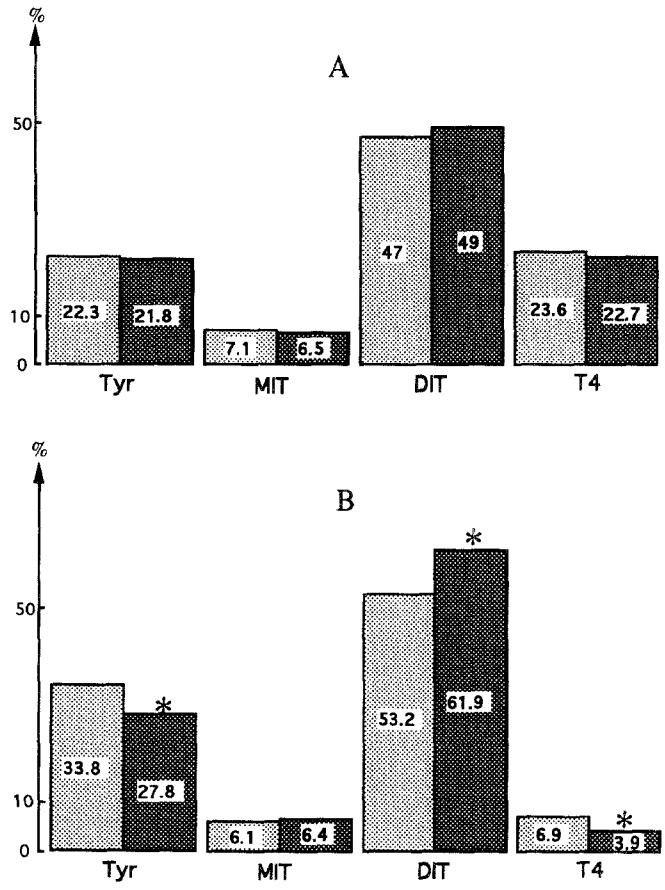

Fig. 2. Radioactivity distribution in L-Tyr, MIT, DIT and T4. **A** LPO-catalyzed iodination. **B** TPO-catalyzed iodination. Experimental procedure: see Materials and methods; *: $p < 0.05$; ▭ Tyr; ▩ Tyr + Tyr-P

Discussion

In vivo experiments with the phosphonic analogue of tyrosine should be paralleled to similar assays with the natural aminocarboxylic acid; experiments with tyrosine have been consequently performed, but the very low solubility of this compound in aqueous media limited the quantity deposited on the chicken embryo to 2 μmoles. Using this latter dose, no effect could be noted on mortality, weight or iodine uptake by thyroid.

Solubility is not a limiting factor for DL-Tyr-P, a much more hydrophilic compound, as doses up to 30 μmoles can be experimented. We were thus able to show the influence of Tyr-P on both mortality rate and on iodine uptake. The marked effect on mortality is partially due to a non specific action of the ionic strength. On the other hand, the significant reduction of iodine uptake may indicate a specific influence of Tyr-P, which can prevent the incorporation of the labeled iodine into the thyroid. The iodination of the tyrosine phosphonic analogue could compete for different steps of iodine fixation on the tyrosyl residues of thyroglobulin.

To support this hypothesis, we carried out kinetic studies of the *in vitro* iodination of DL-Tyr-P using lacto and thyroid peroxidases, two enzymes of different specificity. Beside tyrosine, both peroxidases catalyzed the iodination of Tyr-P and of the four tyrosine analogues tested.

No significant differences in the kinetic parameters of Tyr stereoisomers L and D were observed, a result different from the data reported by Baysse et al. (1972). Lactoperoxidase recognizes Tyr and its phosphonic analogue as substrates but the affinity for L-Tyr is seven times higher, whereas the V_{max} values are similar for both molecules. Compared with the other analogues tested for their ability to be iodinated by LPO, Tyr-P appeared to be a weak substrate. With thyroid peroxidase, Tyr and Tyr-P differ both in their affinity for the enzyme (three times lower for Tyr-P) and in their Vmax (more than three times higher for Tyr-P than for Tyr).

Thus, among Tyr analogues, Tyr-P deserves a special attention, as it hampers iodotyrosine formation, like α-methyl-Tyr (De Groot and Jaksina, 1969). The higher iodination rate of the phosphonate by TPO compared to that of the natural substrate remains to be elucidated. The occurrence of a contaminant iodotyrosine deiodinase in our hog thyroid extract may support a partial explanation; a similar deiodinase has been described in the Rat (Michel et al., 1969). We have observed that the incubation of DIT with our hog enzymatic preparation led to MIT formation (unpublished data). Demonstration of the inertia of the iodo derivatives of Tyr-P towards such enzymatic deiodination would confirm our hypothesis. To conclude, the phosphonic analogue of Tyr could interfere in thyroid hormonogenesis as an iodine trap.

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