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INVESTIGATIONS INTO LACTOPEROXIDASE-CATALYSED BROMINATION OF TYROSINE AND THYROGLOBULIN

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SUMMARY

Thyroid peroxidase and lactoperoxidase are capable of producing oxidized bromine species. Thus investigations into bromination reactions with tyrosine and thyroglobulin were undertaken in order to gain insight into possible formation of brominated thyroid hormone analogues. A reversed-phase high-performance liquid chromatographic method was developed for the separation of bromine/iodine-substituted tyrosines and used as a basis for these investigations combined with ultraviolet absorption and electrochemical detection. The results indicate that in vivo bromination of tyrosyl residues in thyroglobulin might be of some importance in cases of either iodine deficiency or excessive bromide intake.

INTRODUCTION

Peroxidase-catalysed iodination reactions are known to be important in the first step of the biosynthesis of the thyroid hormones triiodothyronine and tetraiodothyronine: thyroid peroxidase catalyses the iodination of tyrosyl residues of thyroglobulin by iodide and hydrogen peroxide in the thyroid gland. In vitro experiments indicated that peroxidases, such as thyroid peroxidase and lactoperoxidase, are also able to utilize bromide as a substrate [1,2]. Thus thyroglobulin bromination and the occurrence of brominated or mixed iodinated and brominated thyronines might be postulated in situations of elevated bromide intake; such situations might occur during balneotherapy with iodide- and bromide-containing brines. Brominated thyronines are known to exhibit hormone activity similar to that of the thyroid hormones triiodothyronine and tetraiodothyronine [3], but their existence in the organism has not yet been proved. Nevertheless, pharmacological and toxicological studies show that bromide can exert changes on the status of the thyroid gland: bromide intake appears to suppress the pro-

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duction of the thyroid hormone triiodothyronine [4,5], which might be explained by the hypothesis that bromide competes with iodide for the halogenation of tyrosyl residues of thyroglobulin. It has been stressed, however, that conclusive evidence may be obtained only by isolation and analysis of all thyronine derivatives from a bromide-treated organism [4,5], which until now has not been done.

To get more insight into the action of bromide and iodide during thyroid hormone biosynthesis, we conducted investigations of the peroxidase-catalysed in vitro formation of bromine/iodine-substituted tyrosines (i.e. 3-bromotyrosine, 3,5-dibromotyrosine, 3-iodotyrosine, 3,5-diiodotyrosine, 3-bromo-5-iodotyrosine) from tyrosine and thyroglobulin. For these investigations a quantitative evaluation of different iodo/bromotyrosines was necessary. Several high-performance liquid chromatographic (HPLC) separations of iodotyrosines and iodothyronines have been reported in the literature [6–11], but these separations have not yet been extended to brominated or mixed brominated and iodinated tyrosines. Now an adequate HPLC method has been developed with UV absorption and amperometric detection in series to obtain the necessary selectivity and sensitivity.

EXPERIMENTAL

Instrumentation and reagents

The chromatographic apparatus consisted of a Waters M510 HPLC pump, a Rheodyne 7125 injection valve with a 20-μl loop and a Merck glass cartridge separation column (150 mm$\times$3 mm I.D.) with a guard column (30 mm$\times$3 mm I.D.), both filled with 5-μm LiChrosorb RP18. A Waters M481 UV–Vis detector and a Metrohm 611 VA detector with a wall-jet cell equipped with glassy carbon as working electrode, Ag/AgCl/3 *M* KCl as reference electrode and gold as auxiliary electrode were used.

The mobile phase was prepared by mixing 80 ml of ethanol with 350 ml of 0.2 *M* phosphoric acid and 600 ml of 0.2 *M* sodium acetate buffer (pH 4.5) and adding 2.74 g of sodium dodecylsulphate.

The flow-rate of the mobile phase was 0.7 ml/min, the detection wavelength was 280 nm and the detection potential of the electrochemical detector was 1000 mV versus Ag/AgCl/3 *M* KCl.

Lactoperoxidase (from cow milk) was supplied by Boehringer-Mannheim (Mannheim, F.R.G.) and *Streptomyces griseus* neutral proteinase (pronase E) by Serva (Heidelberg, F.R.G.). Thyroglobulin, 3,5-diiodotyrosine, 3-iodotyrosine and 3,5-dibromotyrosine were obtained from Sigma (St. Louis, MO, U.S.A.). 3-Bromotyrosine was synthesized by treating a solution of 5.5 mmol of tyrosine in 30 ml of acetic acid and 15 ml of 2 *M* hydrochloric acid with 6.1 ml of acetic acid containing 3.9 mmol of bromine. Excess bromine was reduced by addition of a small amount of sodium bisulphite. The reaction solution was neutralized and used as stock solution (stored refrigerated) without isolation of 3-bromotyrosine. The yield of the synthesis was determined by HPLC analysis of the by-product 3,5-dibromotyrosine and the starting product tyrosine.

For the synthesis of 3-bromo-5-iodotyrosine, 5 ml of a 14 m*M* 3-bromotyrosine

solution were mixed with 5 ml of 33% ethanolic methylamine; this mixture was treated with a slight excess of 1 *M* potassium triiodide. After addition of a small amount of sodium bisulphite, the mixture was neutralized and used as stock solution (stored refrigerated) for the further experiments; the yield was again determined by HPLC.

Lactoperoxidase-catalysed halogenation

A 1-ml volume of a 20 m*M* phosphate buffer (pH 5) containing 2.2 μmol of tyrosine or 1 mg of thyroglobulin was mixed with 10 μl of 2 *M* potassium bromide containing various amounts of potassium iodide; 5 μl of 0.5 *M* hydrogen peroxide and 5 μl of a lactoperoxidase suspension (1000 U/ml) were added. The mixture was allowed to react for 5 min at room temperature. In the case of tyrosine halogenation the mixture was used for HPLC analysis without further treatment; for thyroglobulin halogenation 1 ml of the reaction solution was mixed with an equal volume of hydrolysing reagent (5 mg of pronase in 10 ml of 0.05 *M* borate buffer, pH 8.5) and 0.5 ml of 0.1 *M* sodium hydroxide and left to react for 20 h at 37°C. After adjustment of the pH to 4, the mixture was used for HPLC.

RESULTS AND DISCUSSION

Among the various HPLC methods for the separation of non-derivatized amino acids, the use of reversed-phase chromatography and addition of alkyl sulphate salts or alkyl sulphonate salts to the mobile phase [12–16] is an attractive approach. Such an elution system can easily be tuned to special separations by optimizing the pH and the amount of organic modifier. Therefore a chromatographic method of this kind was chosen for the separation of iodine- or bromine-containing tyrosines. The amino acids tryptophan and iodo/bromo-substituted thyronines were included into the optimization procedure, because these compounds occur in biological materials to be analysed and are detected by both the UV and the electrochemical detector. Therefore they may give rise to interferences.

An absorption maximum between 280 and 295 nm was typical for all iodo/bromotyrosines investigated in this study; 280 nm was chosen as detection wavelength, which was an optimum for bromo-substituted tyrosines. Though the sensitivity would be better in a range below 230 nm, detection at such short wavelengths was considered to be of less use with respect to selectivity in the analysis of complex biological matrices. The hydrodynamic voltammograms of the different iodo/bromotyrosines showed half-wave potentials between 780 and 870 mV (monosubstituted tyrosines had higher half-wave potentials than disubstituted tyrosines, brominated tyrosines higher half-wave potentials than iodinated tyrosines) and reached a plateau between 900 and 1000 mV, which therefore was chosen as a detection potential. The electrochemical behaviour may be attributed to a one-electron oxidation of the phenolic hydroxyl group to a free radical intermediate.

A chromatogram of a standard mixture under optimized mobile phase conditions is given in Fig. 1. The limits of detection (amount injected giving a signal-to-noise ratio of 3:1) of the iodo/bromotyrosines with electrochemical detection

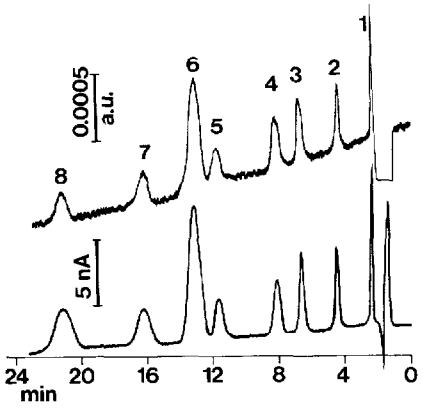

Fig. 1. Separation of substituted tyrosines by HPLC (upper trace: UV detection at 280 nm; lower trace: electrochemical detection at 1000 mV). Peaks: 1 = tyrosine (41 pmol); 2 = 3-bromotyrosine (38 pmol); 3 = 3-iodotyrosine (59 pmol); 4 = 3,5-dibromotyrosine (65 pmol); 5 = 3-bromo-5-iodotyrosine (67 pmol); 6 = tryptophan (78 pmol); 7 = 3,5-diiodotyrosine (145 pmol); 8 = thyronine (80 pmol).

and UV detection were 2 and 12 pmol for 3-bromotyrosine, 3 pmol and 16 pmol for 3-iodotyrosine, 5 and 21 pmol for 3,5-dibromotyrosine, 8 and 31 pmol for 3-bromo-5-iodotyrosine and 17 and 55 pmol for 3,5-diiodotyrosine. The method was shown to be linear for at least two orders of magnitude (beginning at the detection limit); for all bromo/iodotyrosines the correlation coefficient (r) was better than 0.9990 ($n=6$) when using the electrochemical detector, and better than 0.9995 ($n=6$) when using the UV detector.

Iodo/bromo-substituted thyronines eluted at capacity factors larger than 25; if such late-eluting compounds are expected in a sample, the column should be flushed with a mobile phase of increased ethanol content for several minutes after the elution of 3,5-diiodotyrosine.

Though the in vitro experiments described in this paper are to be seen in relation to thyroid hormone biosynthesis, lactoperoxidase was used rather than thyroid peroxidase, because under physiological conditions the catalytic behaviours of lactoperoxidase and thyroid peroxidase compare well [17,18] and, unlike thyroid peroxidase, lactoperoxidase is commercially available. Typical chromatograms of lactoperoxidase-catalysed halogenation experiments of tyrosine are shown in Fig. 2. The amount of different iodo/bromo-substituted tyrosines as a function of the iodide/bromide ratio in the reaction mixture is shown in Fig. 3. The bromide concentration was constant in all these experiments and was chosen with respect to an optimal bromination yield. As can be seen from this figure, iodination is much more favoured than bromination; at the highest iodide concentration in Fig. 3, which is only about one tenth of the bromide concentration, brominated compounds have diminished whereas iodinated or mixed iodinated and brominated compounds dominate. These data should be regarded only as a rough estimate of the different kinetics of bromination and iodination. Somewhat unexpected is the result that the amount of 3,5-dibromotyrosine increases when the iodide/bromide ratio is increased. This phenomenon might be explained by the fact that lactoperoxidase also catalyses the oxidation of iodide to hypohalous

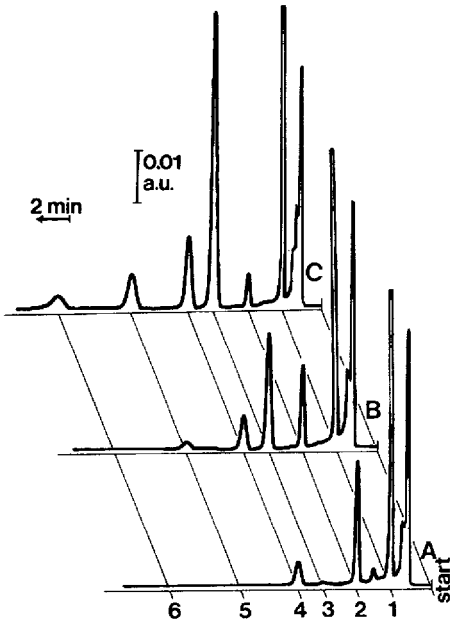

Fig. 2. Typical chromatograms of lactoperoxidase-catalysed bromination and iodination of tyrosine (UV detection at 280 nm). Peaks: 1 = tyrosine; 2 = 3-bromotyrosine; 3 = 3-iodotyrosine; 4 = 3,5-dibromotyrosine; 5 = 3-bromo-5-iodotyrosine; 6 = 3,5-diiodotyrosine: (A) Halogenation with bromide; (B) halogenation with bromide/iodide, 1:0.01 molar ratio; (C) halogenation with bromide/iodide, 1:0.03 molar ratio.

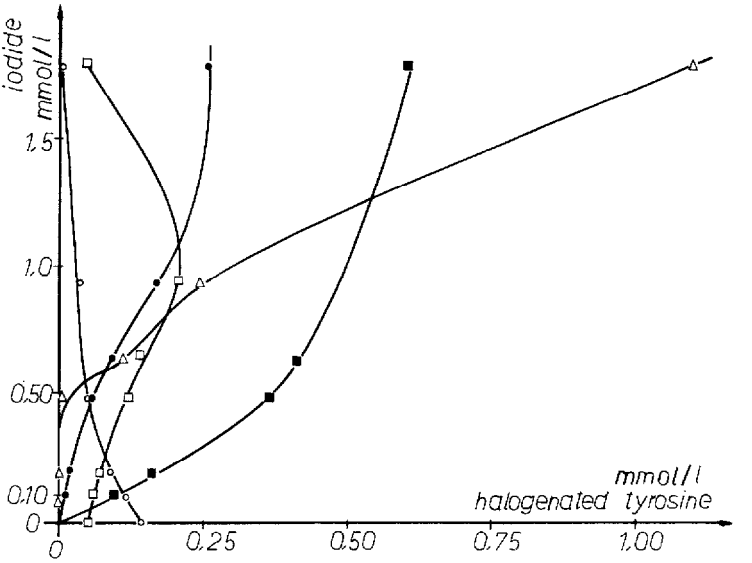

Fig. 3. Formation of different iodo/bromotyrosines as a function of iodide concentration at fixed bromide concentration (19.6 m*M* bromide): (○) 3-bromotyrosine; (□) 3,5-dibromotyrosine; (●) 3-bromo-5-iodotyrosine; (■) 3-iodotyrosine; (∧) 3,5-diiodotyrosine.

acid [1], which in the presence of bromide might form small amounts of the interhalide compound iodine bromide (IBr). The bromination activity of this compound might be greater than that of the peroxidase–hydrogen peroxide–bromide system.

Results similar to those for tyrosine were obtained from experiments with thyroglobulin, as can be seen from Fig. 4, which represents chromatograms of pronase-hydrolysed thyroglobulin. Nevertheless, in the case the situation is somewhat more complicated than with tyrosine. When chromatograms A and D of Fig. 4 are compared, it is evident that bromination leads to a decrease in the content of both 3-iodotyrosyl and 3,5-diiodotyrosyl residues in thyroglobulin, probably because peroxidases are able to catalyse halogen exchange in iodinated tyrosyl residues [19]. Furthermore, some of the added bromide or iodide will be consumed by halogenation of tryptophan [2], as can be seen in Fig. 4 from the diminuation of tryptophan when bromination or iodination is carried out. It may be assumed that halogenated tryptophan species show a chromatographic behaviour similar to that of halogenated thyronines. This means elution at high capacity factors, so that they are not detected under the chosen chromatographic conditions.

From the results of the in vitro experiments described in this paper one may conclude that the formation of bromine-substituted tyrosyl residues in thyroglobulin is possible and that therefore the existence of brominated thyroid hormone analogues cannot a priori be neglected. With respect to the in vivo situation

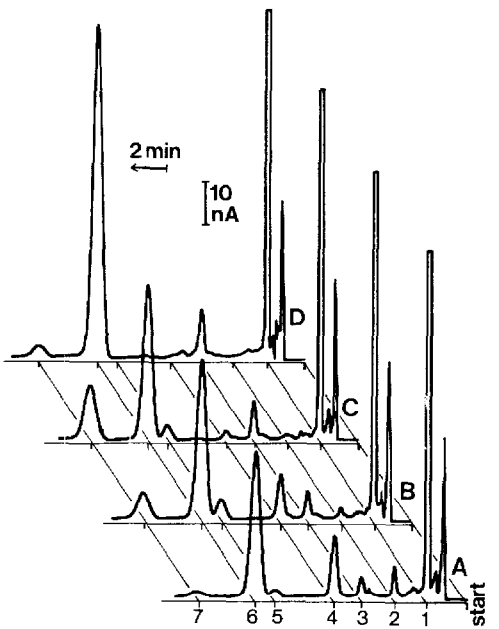

Fig. 4. Chromatograms of hydrolysates of lactoperoxidase-catalysed brominated and iodinated thyroglobulin (electrochemical detection at 1000 mV). Peaks: 1 = tyrosine; 2 = 3-bromotyrosine; 3 = 3-iodotyrosine; 4 = 3,5-dibromotyrosine; 5 = 3-bromo-5-iodotyrosine; 6 = tryptophan; 7 = 3,5-diiodotyrosine. (A) Halogenation with bromide; (B) halogenation with bromide/iodide, 1:0.01 molar ratio; (C) halogenation with bromide/iodide, 1:0.03 molar ratio; (D) without halogenation.

it may be postulated that if these compounds are biosynthesized in the thyroid gland they will be formed in situations of high bromide/iodide ratio, caused by either iodine deficiency or excessive bromide intake. Work on the uptake of bromide and iodide by the thyroid gland, as well as on the analysis of brominated tyrosyl residues in the thyroid tissue, is in progress. Preliminary experiments [20] indicate that the HPLC method described in this paper is also applicable to such samples. When analysing biological samples, the electrochemical detector showed better selectivity than the UV detector, so in general quantitative data were taken from the electrochemical rather than from the UV detector, though both detectors were always coupled in series to the separation column.

Continuation of the work outlined in this paper will necessitate the development of an appropriate separation system for brominated and mixed brominated and iodinated thyronines.

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