Re-examination of the Subcellular Localization of Thyroxine 5'-Deiodination in Rat Liver

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(Received 25 September 1978)

We describe the existence of at least two thyroxine 5'-deiodinases in rat liver. They co-fractionate with NADPH-cytochrome *c* reductase, the marker enzyme for membranes of the endoplasmic reticulum. Subcellular-localization studies of the most active microsomal thyroxine 5'-deiodinase were performed under substrate saturation and at optimal pH 6.8. This enzyme has a $K_{m(\text{app.})}$ of about $3\ \mu\text{M}$ -thyroxine and a V_{max} of about 8 ng of tri-iodothyronine/min per mg of protein. Our study confirms in part the earlier reports of microsomal localization of thyroxine 5'-deiodination. However, this process is not mediated by only a single enzyme.

Thyroxine is the main secretory product of the thyroid gland. Extrathyroidal deiodination of thyroxine at the phenolic ring leads to 3,3',5'-tri-iodothyronine, which is thought to be the main metabolically active hormone, whereas 3,3',5'-tri-iodothyronine, which arises from deiodination at the tyrosine ring of thyroxine, seems to exhibit a regulatory function. 3,3',5'-Tri-iodothyronine is further deiodinated to 3',5'-di-iodothyronine and 3,3'-di-iodothyronine (Kaplan & Utiger, 1978; Chopra, 1977; Höffken *et al.*, 1977). Chopra (1977) demonstrated that 3',5'-di-iodothyronine has a similar ability to inhibit thyroxine deiodination to that of 3,3',5'-tri-iodothyronine. 3,3'-Di-iodothyronine is an intermediate product, which is derived from tri-iodothyronine and 3,3',5'-tri-iodothyronine (Höffken *et al.*, 1977; Hüfner & Grussendorf, 1978).

Thyroxine 5'-deiodination, which is enzymic, was mainly investigated in rat liver and kidney tissues by radioimmunochemical determination of tri-iodothyronine. Initially, the subcellular localization of this process was ascribed to the microsomal fraction of rat liver (Hesch *et al.*, 1975). In some later reports this result was confirmed, and in others it was

contradicted (see Table 1). These discrepancies stimulated us to re-examine the subcellular localization of thyroxine 5'-deiodination. It was our intention to pay particular regard to the principles of tissue fractionation and subcellular localization given by de Duve (1964, 1965). For example: (1) the subcellular fractions were characterized by marker enzymes and by the sedimentation coefficients of their particles (de Duve & Berthet, 1953); (2) the centrifugation (de Duve & Berthet, 1953) and homogenization techniques were standardized; (3) maximal enzyme activities were measured at optimal conditions; (4) for kinetic experiments we detected initial velocities, i.e. enzyme activities were linear with time and protein concentration. Finally, we have investigated the pH-dependence of thyroxine-5'-deiodination in several cell fractions.

Experimental

Iodothyronines were a gift from Henning Berlin G.m.b.H., Berlin, Germany. Other reagents were of the highest purity commercially available.

Table 1. Previous subcellular localization of thyroxine 5'-deiodination in rat liver or kidney

Reference	Organ investigated	Subcellular fraction	Marker enzymes measured	Thyroxine 5'-deiodination saturated with thyroxine in incubations	Thiol compounds added
Hesch <i>et al.</i> (1975)	Liver	Microsomal	Not published	No	No
Visser <i>et al.</i> (1976)	Liver	Microsomal	Glucose 6-phosphatase	No	Yes
Chopra (1976)	Liver	Mitochondrial	—	?	No
Cavalieri <i>et al.</i> (1977)	Liver	Microsomal, cytosol	—	No	Yes
Leonard & Rosenberg (1977)	Kidney	Plasma membrane	5'-Nucleotidase, (Na ⁺ + K ⁺)-dependent ATPase	?	Yes

Adult male Sprague-Dawley rats were decapitated and bled after 12–24 h of food deprivation. For some experiments rats were anaesthetized by intraperitoneal injection of 12.5–20 mg of ketamine hydrochloride (Vetalar; Parke, Davis and Co., München, Germany) per 100 g body wt., and then injected with 115 µg of heparin (Thrombophob; Nordmark-Werk G.m.b.H., Hamburg, Germany) per 100 g body wt. Then the livers were perfused *in situ* with 100 ml of 0.9% NaCl containing 3.1 mg of heparin via the portal vein to free the organ of blood.

The animals were injected with Triton WR-1339 (Leighton *et al.*, 1968), if a modification of the centrifugation scheme described by Leighton *et al.* (1968) was to be used for fractionation (for characteristics of cell fractions see Table 5). The minced livers were washed in ice-cold buffer A (0.25 M-sucrose, 3 mM-dithioerythritol, 3 mM-EDTA, 10 mM-Tris/HCl, pH 7.4) and homogenized in 9 vol. of buffer A in a Potter homogenizer (Braun, Melsungen, Germany) with three strokes at 400 rev./min. The pellets were rehomogenized with 1 stroke at 150 rev./min.

For density-gradient centrifugation 3 ml of the large-granular pellet (P₄), which contains most of the lysosomes, peroxisomes and mitochondria, was layered over 32 ml of a linear sucrose gradient (25–30 to 50–55%, w/w). Centrifugation conditions were: 90 min at 23 000 rev./min, r_{min} about 90 mm, r_{max} 165 mm; 6 × 38 ml MSE aluminium swing-out rotor, 4°C.

Conventional differential centrifugation modified as described by Schneider (1948) was used to compare our results with previous data (Hesch *et al.*, 1975; Chopra, 1976; Visser *et al.*, 1976).

Cell fractions were investigated immediately, or after storage at –20°C. In a total volume of 400 µl, 20–2000 µg of cell fraction protein was incubated with 0.065–30 µM-thyroxine in buffer B [90 mM-tetraethylethylenediamine (Semenza *et al.*, 1962), 63 mM-Tris/HCl, pH 5.5–8.5, 3 mM-EDTA, 3 mM-

dithioerythritol] for up to 20 min at 37°C. The reaction was stopped and the iodothyronines were extracted with 800 µl of ice-cold ethanol. After shaking for 15 min the incubation mixture was centrifuged at 10 500 g for 8 min at 4°C (Mikroliter Zentrifuge, Hettich, Tuttlingen, Germany). Tri-iodothyronine was determined by radioimmunoassay in ethanolic supernatants (Hesch *et al.*, 1974). Zero-time incubations were performed by adding the cell fractions to the reaction mixture after the ethanol. Tri-iodothyronine detected in these incubations was attributed to iodothyronine contamination of the thyroxine substrate cross-reacting with the anti-(tri-iodothyronine) serum. In control incubations in which no substrate was added, no endogenous tri-iodothyronine could be detected. Values for zero-time and control incubations were independent of the addition of different cell fractions. Control values were identical with those for the specific binding of tri-iodothyronine in the radioimmunoassay. We therefore assumed that the specific reaction of the ethanolic cell extract and that of the standards with the antiserum were identical in the tri-iodothyronine radioimmunoassay.

Seven marker enzymes (see Table 4) were measured by commonly used methods; NADPH-cytochrome *c* reductase (Sottocasa *et al.*, 1967); glucose 6-phosphatase [Swanson, 1950, as modified by Hübscher & West (1965) as well as by Leighton *et al.* (1968)]; succinate dehydrogenase (Brdiczka *et al.*, 1968); lactate dehydrogenase (Bergmeyer & Bernt, 1974); acid phosphatase [Leighton *et al.* (1968), but with *p*-nitrophenol as substrate as described by Walter & Schütt (1974)]; 5'-nucleotidase (Ebel *et al.*, 1976); uricase [Bergmeyer (1974) but with 0.1% Triton X-100 in the incubation mixtures as described by Leighton *et al.* (1968)]. Protein was determined by the biuret (Bode *et al.*, 1968) and the Lowry (Lowry *et al.*, 1951) procedures, the latter with the addition of a constant quantity of dithioerythritol in standards and blanks to account for the contents of thiol groups.

Table 2. Dependence of thyroxine 5'-deiodination in homogenate on protein concentration
Incubation mixture consisted of buffer B, pH 7.4, and 1 µM-thyroxine at 37°C.

Protein in incubation vessel (mg)	Time of incubation (min)	Tri-iodothyronine formed (pg/min per mg)
0.43	10	899 ± 99
0.215	10	1429 ± 43
0.108	10	1600 ± 128
0.043	10	1619 ± 340
0.022	10	1200 ± 324
0.011	10	873 ± 873
0.43	20	988 ± 10
0.215	20	1362 ± 27
0.108	20	1533 ± 92
0.043	20	1702 ± 119
0.022	20	1636 ± 278
0.011	20	1963 ± 550

We used a control serum (Labtrol; Merz & Dade G.m.b.H., München, Germany) as standard.

Activities are given as means $\pm$ s.d. in the Tables and Figures. Significances were obtained by Student's *t* test.

Results and Discussion

Effects of storage of cell preparations

Thyroxine-5'-deiodination activities in cell fractions containing 3mM-dithioerythritol did not decrease for up to 6 weeks, when kept at -20°C . After 3 months, activities had fallen to 45%. Correspondingly, NADPH-cytochrome *c* reductase activities decreased from 100 to 31%.

Dependence of thyroxine 5'-deiodination on protein concentration

Experiments for subcellular localization of thyroxine 5'-deiodination varied with incubation time and were performed with $1\mu\text{M}$ - or $10\mu\text{M}$ -thyroxine. Thyroxine 5'-deiodination was linear with time (Table 2). Table 2 demonstrates further that specific thyroxine 5'-deiodination increased with decreasing protein concentration and reached a range in which it was independent of protein concentration. This result confirms those of Leonard & Rosenberg (1977). The protein-dependence of specific thyroxine 5'-deiodination was more pronounced in crude than in pure cell fractions, e.g. microsomal. Therefore relative specific activities of thyroxine 5'-deiodination in purer cell fractions were higher than those of corresponding marker enzymes. This holds true for experiments performed without substrate saturation (e.g. $1\mu\text{M}$ -thyroxine) and at a protein concentration at which thyroxine 5'-deiodination was not independent of the protein amount (Table 1). Therefore we have proved independence of relative specific activities of thyroxine 5'-deiodination from protein concentration at $1\mu\text{M}$ -thyroxine (Table 2). At low protein concentrations, however, the experimental error increased considerably.

We assume that thyroxine binds to sites different from thyroxine 5'-deiodinases. This is supported by experimental data demonstrating the dependence of thyroxine 5'-deiodination on the concentration of human albumin present in the reaction mixture. For example, on the addition of 1mg of albumin to $430\mu\text{g}$ of cellular protein/incubation tube the activity of thyroxine 5'-deiodination decreased from 1ng of tri-iodothyronine/min per mg to 0.1ng of tri-iodothyronine/min per mg of protein.

Kinetic investigations

Fig. 1 shows the substrate saturation of thyroxine-5'-deiodination activity. If, however, the first steep

part of this curve is analysed, several plateaus can be observed (Figs. 2, 3 and 4). We studied the kinetic data reported by Hesch *et al.* (1975), Visser *et al.* (1975) and Chopra (1977) and discovered the same phenomenon of intercurrent plateau formation. Such kinetics are found, if several enzymes catalysing the same reaction are active. Plotting our kinetic data of v/s^2 dependent on v or of v/s^3 dependent on v we can calculate three kinetic constants for thyroxine 5'-deiodination (Table 3). We interpret the results as showing that there are at least two thyrox-

Fig. 1. Saturation of thyroxine-5'-deiodination activity in a microsomal fraction

Incubations were performed at pH 7.6 for 10 min at 37°C in buffer B (but with 21mM-Tris/HCl and 30mM-tetraethylethylenediamine). For further details see the text.

Fig. 2. Kinetics of thyroxine-5'-deiodination activity in a microsomal fraction

The same conditions as for Fig. 1 were used, except for pH, which was 6.9. For further details see the text.

Fig. 3. Kinetics of thyroxine-5'-deiodination activity in a microsomal fraction
Incubation was in buffer A, pH7.4, for 10min at 37°C. For further details see the text.

ine-5'-deiodinating processes. Another possible explanation for the non-Michaelis-Menten-like kinetics is the occurrence of allosteric effects (Wong, 1975). Thyroxine itself and probably its metabolites, e.g. 3,3',5'-tri-iodothyronine and 3',5'-di-iodothyronine, whose production depends on the pH, might contribute to this behaviour.

pH-dependence

In repeated experiments two peaks of thyroxine-5'-deiodination activity were observed in the microsomal fraction, as demonstrated in Fig. 5. In the large-granular fraction the second peak at the low pH was not found. There are two possible explanations for this. (1) Two microsomal thyroxine 5'-deiodinases with different pH optima do exist (see also I and II in Table 3). (2) The finding can also be simulated by pH-dependent degradation of tri-iodothyronine to substances different from 3,3'-di-iodothyronine, or by pH-dependent production of thyroxine-5'-deiodination-inhibiting iodothyronines.

Fig. 4. Kinetics of thyroxine-5'-deiodination activity in a microsomal fraction
More substrate concentrations than for Fig. 1 were investigated and a freshly prepared microsomal fraction was used that was rich in lysosomes. Incubations were performed at pH7.6 (○) and pH6.1 (●) at 37°C in buffer B for 16min. For further details see the text.

A clear-cut explanation will be possible after isolation of different thyroxine 5'-deiodinases.

The pH optimum near 6.8 that we describe for the microsomal fraction is comparable with data for tissue homogenates given by Chopra (1977) and Cavalieri *et al.* (1977).

The low-activity plateau of thyroxine 5'-deiodination (Table 3 and Fig. 4) implies the existence of a third thyroxine 5'-deiodinase. It is particularly found in microsomal fractions rich in lysosomes and at acidic pH. Höffken *et al.* (1976) and Visser *et al.* (1978) reported pH optima of 5.5 and 6–6.5.

The existence of several thyroxine 5'-deiodinases, the different experimental conditions used by various investigators for, e.g. characterization of subcellular preparations and their storage, the use of different substrate concentrations and thiol-group-protecting agents and the distribution of experimental points

Table 3. Apparent characteristics of the three thyroxine-5'-deiodinating processes

Thyroxine 5'-deiodinase	K_m (μ M-thyroxine)	V_{max} . (pg of tri-iodothyronine/min per mg)	pH optimum	Subcellular location
I	2–3.5	8000	6.8	Microsomal
II	1	2000–3000	5.8–6.3	Microsomal
III	0.2–0.6	200–300	5–6	?

along the pH range (Chopra, 1977) all help to explain why the pH optima so far published differ considerably. These different experimental conditions

may also explain why the pH optimum of only one of the postulated thyroxine 5'-deiodinases has previously been discovered.

Subcellular localization

The results of our localization experiments are summarized in Table 4. The experiments were performed with 1 μM- or 10 μM-thyroxine in the pH range 6–7.6. With respect to subcellular localization, we can find no essential differences dependent on pH or substrate. Localization of thyroxine 5'-deiodination correlates with microsomal marker enzymes only. Enrichment of thyroxine 5'-deiodination and the microsomal marker enzyme activities compared with the homogenate is 4–6-fold in microsomal fractions.

Recoveries of enzyme activities have not generally been satisfactory. However, the recoveries of thyroxine-5'-deiodination and microsomal marker enzyme activities correlate very well at the different points of subcellular fractionation. This confirms the microsomal localization of thyroxine 5'-deiodination.

Table 5 shows the results of a typical localization experiment with fresh cell fractions. Thyroxine-5'-deiodination activities correlated well with NADPH-cytochrome c reductase, and *s*_{0.5} *V*_{max}. (see Table 5) was lowest in the microsomal fractions.

Of fractions prepared by density-gradient centrifugation at pH7.2 only the microsomal fraction showed significant thyroxine-5'-deiodination activity. Maximal thyroxine-5'-deiodination activities measured in microsomal fractions were about 8 ng of tri-iodothyronine/min per mg.

Fig. 5. pH-dependences of thyroxine-5'-deiodinating activities in a microsomal and large-granular fraction A microsomal (P₆; ●) and a large-granular fraction (P_{3/4}; ○) were incubated for 10min at 37°C with 2 μM-thyroxine. For further details see the text.

Table 4. Correlation of relative specific activities of marker enzymes to relative specific activities of thyroxine 5'-deiodination Relative specific activity is the specific activity in the fraction relative to that in the homogenate. 'Yes' indicates that a positive correlation of thyroxine 5'-deiodination to marker enzyme exists, 'No' indicates that a positive correlation does not exist. The presence (+) or absence (–) of significant enzyme activity in the subcellular fraction is indicated.

Marker enzyme	Subcellular location	Subcellular fraction			
		600g pellet P _{1/2}	Large-granular P _{3/4}	Microsomal P ₆	Cytosolic S ₆
5'-Nucleotidase (EC 3.1.3.5)	Cell membrane	No +	No +	No +	No +
Lactate dehydrogenase (EC 1.1.1.27)	Cytosol	No +	No +	No +	No +
Succinate dehydrogenase (EC 1.3.99.1)	Mitochondria	No +	No +	No –	No –
Acid phosphatase (EC 3.1.3.2)	Lysosomes (Tritosomes)	No +	No +	No +	No –
Uricase (EC 1.7.3.3)	Peroxisomes	No +	No +	No –	No –
Glucose 6-phosphatase (EC 3.1.3.9)	Microsomes	Yes +	Yes +	Yes +	Yes –
NADPH-cytochrome c reductase (EC 1.6.2.4)	Microsomes	Yes +	Yes +	Yes +	Yes –
Significant thyroxine-5'-deiodination activity present		+	+	+	–

Table 5. Marker enzyme and thyroxine-5'-deiodination activities in subcellular fractions

Incubation mixtures contained protein (between 200 and 500 µg/tube), 10 µM-L-thyroxine and buffer B, pH 6.9; the incubation time was 10 min and the temperature was 37°C. Relative activity is specific activity of enzyme in fraction relative to that in homogenate (100%). $s_{\min}$ is sedimentation coefficient of the smallest completely sedimenting particle prepared by our technique. $s_{0.5} V_{\max}$ is the substrate concentration that is necessary to achieve half-maximal velocity in the cell fractions; results of an experiment at pH 7.4.

Cell fraction	Sedimentation coefficient (S)	Specific activity of enzymes				$s_{0.5} V_{\max}$ (µM-thyroxine)
		NADPH-cytochrome c reductase (munits/mg)	Thyroxine-5'-deiodination (pg of tri-iodothyronine/min per mg)	Relative activity (%)	Relative activity (%)	
Homogenate	—	22.1 ± 1.6	495 ± 147	100 ± 7	100 ± 30	4-5
600g pellet, $P_{1/2}$	$s_{\min} = 331983$	29 ± 0.5	660 ± 113	131 ± 2	133 ± 23	4-5
Post-600g supernatant, $S_{1/2}$	$s < 331983$	20.4 ± 3.2	443 ± 256	92 ± 14	89 ± 52	3-4
Large-granular pellet, $P_{3/4}$	$s_{\min} = 2850$	25.7 ± 1.3	398 ± 78	116 ± 6	80 ± 16	4-5
Post-large-granular supernatant, $S_{3/4}$	$s < 2850$	16.6 ± 0.8	108 ± 85	75 ± 4	22 ± 17	—
Heavy-microsomal pellet, P_5	$s_{\min} = 2850$	90.5 ± 8.4	2004 ± 124	410 ± 38	405 ± 25	2-3
Post-heavy-microsomal supernatant, S_5	$s < 2850$	15.2 ± 3.4	435 ± 133	69 ± 15	88 ± 27	3-4
Microsomal pellet P_6	$s_{\min} = 50$	39.8 ± 4.9	1122 ± 152	180 ± 22	227 ± 31	2-3
Cytosol S_6	$s < 50$	0.8 ± 1.1	—	4 ± 5	—	—

Conclusions

Our experiments suggest that there are at least two thyroxine 5'-deiodinases in rat liver, as summarized in Table 3. The most active (I) is localized in microsomes and has a K_m of about 3 µM for thyroxine, a $V_{\max}$ of about 8 ng of tri-iodothyronine/min per mg and a pH optimum of 6.8. The second thyroxine 5'-deiodinase, which has the following characteristics, is also found in the microsomes: K_m about 1 µM for thyroxine, $V_{\max}$ 2-3 ng of tri-iodothyronine/min per mg, pH optimum about 6. The existence of the third thyroxine 5'-deiodinase is postulated from kinetic data of thyroxine 5'-deiodination in microsomal fractions that are rich in lysosomes. Thyroxine 5'-deiodinase III has the lowest activity.

We have localized thyroxine 5'-deiodinase, which forms 3,3',5'-tri-iodothyronine, and 3,3',5'-tri-iodothyronine 3'-deiodinase and 3,3',5'-tri-iodothyronine 3-deiodinase, both of which form 3,3'-di-iodothyronine in microsomes (D. Auf dem Brinke, J. Köhrle, R. Ködding & R. D. Hesch, unpublished work). Therefore we suggest that these iodothyronine-deiodinating processes are part of a microsomal enzyme complex. This metabolic pathway of thyroxine seems to be regulated by the concentration of reduced glutathione [as is assumed by Visser *et al.* (1978)], by the pH and by iodothyronines (Höfken *et al.*, 1977). The postulated heterogeneity of thyroxine 5'-deiodinases as well as the other iodothyronine-deiodinating enzymes must conclusively be demonstrated by isolating these proteins.

This work is part of a dissertation by D. A. d. B.

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