Subcellular Localization of a Rat Liver Enzyme Converting Thyroxine into Tri-iodothyronine and Possible Involvement of Essential Thiol Groups

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Experiments with rat liver homogenates showed that on subcellular fractionation the ability to catalyse the conversion of thyroxine into tri-iodothyronine was lost. The activity could in part be restored by addition of the cytosol to the microsomal fraction. Both components were found to be heat labile. The necessity of the presence of cytosol could be circumvented by incorporation of thiol-group-containing compounds in the medium. Optimal enzymic activity was observed in the presence of dithiothreitol and EDTA in medium of low osmolarity. By comparing the distribution of the converting enzyme over the subcellular fractions with a microsomal marker enzyme, glucose 6-phosphatase, it was demonstrated that the former is indeed of microsomal origin. Finally, it was shown that thiol groups play an essential role in the conversion of thyroxine into tri-iodothyronine.

Soon after the detection of 3,3',5-tri-iodothyronine in human plasma in 1952 by Gross & Pitt-Rivers, it was recognized that circulating tri-iodothyronine was in part derived from peripheral deiodination of thyroxine (Pitt-Rivers *et al.*, 1955; Larson *et al.*, 1955). It was suggested that tri-iodothyronine is the principal biologically active hormone, with thyroxine as a large precursor pool enabling the body to maintain constant effective thyroid hormone concentrations (Gross & Pitt-Rivers, 1954; Schwartz *et al.*, 1971). This pro-hormone function of thyroxine has been substantiated by the finding that its affinity for the nuclear thyroid hormone receptor is small relative to tri-iodothyronine (Oppenheimer *et al.*, 1973; Samuels & Tsai, 1973; Docter *et al.*, 1976).

The study of the conversion of thyroxine into tri-iodothyronine *in vitro* has been hampered by the occurrence of an apparently non-specific deiodination yielding reaction products other than tri-iodothyronine (Tata, 1960; Stanbury *et al.*, 1960; Wynn *et al.*, 1962; Galton & Ingbar, 1963; Nakagawa & Ruegamer, 1967; Schwartz *et al.*, 1969; Dawber *et al.*, 1971). Other investigations by Larson *et al.* (1955), Rabinowitz & Hercker (1971), Sterling *et al.* (1973), Klebanoff & Green (1973) and Woeber & Ingbar (1973) using tissue slices or isolated cells have unequivocally shown that production of tri-iodothyronine as a result of mono-deiodination of thyroxine can be observed.

These observations were extended by the application of specific radioimmunoassays for the measurement of tri-iodothyronine on incubation of tissue

homogenates with thyroxine by Hesch *et al.* (1975), Chiraseveenupapund *et al.* (1975) and Visser *et al.* (1975). The mechanism of the specific deiodination, however, remains unclear. We report in the present paper results of investigations that demonstrate that the conversion of thyroxine into tri-iodothyronine is catalysed by an enzyme of the endoplasmic reticulum of rat liver tissue and that thiol groups are essential for this enzymic activity.

Experimental

Materials

L-Thyroxine, *p*-chloromercuriphenylsulphonic acid and dithiothreitol were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of rat liver homogenate and subcellular fractionation

The post-nuclear supernatant of rat (Wistar) liver homogenate was prepared in 25 mM-Tris/HCl, pH 7.4, containing 0.25 M-sucrose as previously described (Visser *et al.*, 1975). This preparation was fractionated into mitochondrial, lysosomal, microsomal and cytosolic fractions by means of differential centrifugation essentially by the method of de Duve *et al.* (1955). The purity of the fractions thus obtained was evaluated by analysis of several marker enzymes (de Duve *et al.*, 1955). As a microsomal marker glucose 6-phosphatase (EC 3.1.3.9) was measured (de Duve *et al.*, 1955). Protein was measured as described by Lowry *et al.* (1951) with bovine serum albumin as standard.

Table 1. *Tri-iodothyronine production on incubation of thyroxine (1 μ M) with subcellular fractions in 0.25M-sucrose/25 mM-Tris/HCl, pH7.4, at 37°C*

The numbers in parentheses are the protein content (in mg/ml) of the various fractions.

Fraction	Tri-iodothyronine formed (pmol/ml per 30 min)
Homogenate(14.8)	14.9
Mitochondrial(1.45)	0.0
Lysosomal(1.25)	0.3
Microsomal(3.5)	0.3
Cytosolic(9.7)	0.3
Mitochondrial(1.45)+cytosolic(7.2)	1.2
Lysosomal(1.25)+cytosolic(7.2)	1.4
Microsomal(3.5)+cytosolic(7.2)	6.0
Microsomal (after 30 min at 56°C)(3.5)+cytosolic(7.2)	0.2
Microsomal(3.5)+cytosolic (after 30 min at 56°C)(7.2)	0.0

Table 2. *Tri-iodothyronine production on incubation of microsomal fraction (5.45 mg of protein/ml) with thyroxine (1 μ M) in 0.25M-sucrose/25 mM-Tris/HCl, pH7.4, at 37°C after a preincubation for 30 min at 0°C in the presence or absence of thiol-group-containing compounds*

Addition Incubation time (min)...	Tri-iodothyronine formed (pmol/ml)	
	15	30
None	0.4	0.8
1 mM-Dithiothreitol	16.3	27.6
1 mM-2,3-Dimercaptopropanol	7.3	11.9
1 mM- β -Mercaptoethanol	6.9	13.3

Table 3. *Tri-iodothyronine production on incubation of thyroxine (1 μ M) with microsomal preparations (3.8 mg of protein/ml) in Tris buffered media (pH7.4) of different compositions at 37°C*

Dithiothreitol (mM)	EDTA (mM)	Sucrose (M)	KCl (M)	Tri-iodothyronine formed (pmol/ml per 30 min)
0	0	0.25	0	0.5
0	5	0.25	0	5.1
1	0	0.25	0	30.8
1	5	0.25	0	49.2
5	0	0.25	0	33.9
5	0	0.25	0.4	26.3
5	0	0	0	30.2
5	5	0	0	61.3
5	5	0	0.4	19.9

Conversion studies

Thyroxine (1.0 nmol in a final volume of 1 ml), was incubated with various subcellular fractions suspended in buffers of different composition as indicated. The reaction was stopped by the addition of

2 vol. of 95% (v/v) ethanol and the tri-iodothyronine produced was measured by radioimmunoassay (Visser *et al.*, 1975). The thyroxine preparation used showed a relative displacement potency in this radioimmunoassay of less than 0.1%, which represents maximal values for contamination with tri-iodothyronine as well as for actual cross-reaction of thyroxine.

Results

Although conversion of thyroxine into tri-iodothyronine by rat liver homogenate could readily be demonstrated (Visser *et al.*, 1975) this catalytic activity was apparently lost on subsequent fractionation (Table 1). Activity could, however, in part be restored by combining the cytosol with the microsomal fraction and to a lesser degree with the other particulate preparations. The active factor present in the soluble fraction as well as that in the particles was found to be heat labile. Heating either cytosol or the particulate components for 30 min at 56°C strongly decreased the converting activity displayed by the combination of the fractions concerned.

In the course of these experiments we noticed that on standing at 0°C for approx. 4 h the homogenate lost over 50% of its converting activity. In addition a decrease in activity occurred when the homogenate was dialysed for a short time; 5 ml of homogenate, dialysed against 2 litre of 0.25M-sucrose/25 mM-Tris/HCl, pH7.4, for 30 min at 0°C, possessed only 45% activity compared with non-dialysed material.

It was observed that the requirement for cytosol could be circumvented by including a thiol-group-containing compound in the reaction mixture, namely β -mercaptoethanol, 2,3-dimercaptopropanol or dithiothreitol, with the latter being far the most effective (Table 2).

Table 3 demonstrates to what extent tri-iodothyronine accumulated, when thyroxine was incu-

bated with microsomal preparations suspended in media varying in sucrose, EDTA, KCl and dithiothreitol concentrations. Obviously conditions are optimal if both EDTA and dithiothreitol are present. KCl (0.4M) inhibited the reaction.

In the presence of 1 mM-dithiothreitol and 5 mM-EDTA, treatment at 56°C for 30 min still abolished the catalytic activity of the microsomal fraction with respect to thyroxine conversion. This was not caused by accelerated oxidation of added dithiothreitol or cysteine residues in the microsomal proteins; it was rather a matter of denaturation, since subsequent addition of dithiothreitol failed to induce activity.

To investigate the possible involvement of thiol groups, tri-iodothyronine-directed deiodination of

thyroxine was studied by incubating it with a microsomal preparation preincubated with agents known to become covalently linked to cysteine residues (Table 4). In the presence of *p*-chloromercuriphenylsulphonic acid, Hg²⁺ or Ag⁺, tri-iodothyronine generation was almost completely suppressed. This decrease in activity was not due to reactions with dithiothreitol and the consequent decrease in its effective concentration, but rather to substitutions at the thiol groups of the enzyme. Experiments were performed in which the preincubation with the cysteine-directed inhibitors was followed by a second preincubation in the presence of excess of dithiothreitol and in no case was any restoration of activity observed. Table 4 also shows that addition of KCN to the reaction mixture did not affect the amount of tri-iodothyronine generated from thyroxine, rendering the involvement of a prosthetic haem group unlikely.

The monodeiodination of thyroxine by the microsomal fraction was investigated in the presence of millimolar concentrations of nicotinamide-adenine dinucleotides (Table 5). In no single instance was any enhancement of the reaction rate observed.

In Table 6 the subcellular distributions of the converting enzyme (assayed under optimal conditions with respect to EDTA and dithiothreitol concentrations) and glucose 6-phosphatase are compared. The highest tri-iodothyronine production rate is observed with the microsomal fraction.

Discussion

Our observations suggest that rat liver homogenate contains a diffusible factor, the presence of which is a prerequisite for the conversion of thyroxine into tri-iodothyronine. The loss of activity on keeping the homogenate at low temperatures could indicate that the factor(s) involved are inactivated either by proteolytic breakdown or, more likely, by oxidation due to exposure to air. The latter possibility was supported by the finding that inclusion of 1 mM-dithiothreitol in the incubation mixture enhanced the production of tri-iodothyronine from thyroxine as

Table 4. *Tri-iodothyronine production on incubation of thyroxine (1 μ M) with microsomal fraction in 25 mM-Tris/HCl/1 mM-dithiothreitol/5 mM-EDTA, pH 7.4, at 37°C in the presence of thiol-group-blocking reagents or CN⁻*

Addition	Tri-iodothyronine formed (%)
None	100
3 mM- <i>p</i> -Chloromercuriphenylsulphonic acid	0.6
3 mM-Ag ⁺	2.0
3 mM-Hg ²⁺	2.7
1 mM-KCN	97

Table 5. *Tri-iodothyronine production on incubation of thyroxine (1 μ M) with microsomal fraction in 25 mM-Tris/HCl/1 mM-dithiothreitol/5 mM-EDTA, pH 7.4, at 37°C in the presence of possible cofactors*

Addition (1 mM)	Tri-iodothyronine formed (%)
None	100
NAD ⁺	115
NADH	94
NADP ⁺	105
NADPH	78

Table 6. *Specific activities of glucose 6-phosphatase and of the enzyme converting thyroxine into tri-iodothyronine in subcellular fractions*

Tri-iodothyronine production was studied with thyroxine (1 μ M) in 0.25 M-sucrose/25 mM-Tris/HCl/3 mM-dithiothreitol/3 mM-EDTA, pH 7.4, at 37°C.

Fraction	Glucose 6-phosphatase (μ mol of phosphate/min per mg of protein)	Thyroxine-5'-deiodinase (pmol of tri-iodothyronine/min per mg of protein)
Homogenate	0.102	2.90
Mitochondrial	0.036	1.73
Lysosomal	0.181	4.34
Microsomal	0.200	6.46
Supernatant	0.002	0.11

catalysed by the homogenate. In addition, the decrease in activity on aging of the homogenate was less rapid in the presence of dithiothreitol than in its absence. Further, it induced converting activity by the microsomal fraction. The results of experiments described in the present paper are in favour of the assumption that the enzyme is highly sensitive to oxidation. Apparently a condition for the enzyme to display converting activity is that cysteine residues are present in the reduced state. The stimulation by cytosol in the experiments described in Table 1 may have been affected by endogenous glutathione although we cannot firmly exclude additional actions of the supernatant fraction.

The only comparable study on the occurrence of the conversion in the presence of rat liver microsomal fraction is that by Hesch *et al.* (1975). Since these investigators did not incorporate reductants such as thiols in their media, the conversion rate they reported is very low. Under comparable circumstances they observed that less than 0.1% of the thyroxine added was deiodinated to tri-iodothyronine, whereas we found approx. 6% (Table 3).

The results described here may also be compared with observations in other laboratories (e.g. Stanbury *et al.*, 1960; Wynn *et al.*, 1962; Nakagawa & Ruegamer, 1967) on the deiodination of thyroxine by rat liver microsomal fraction, apparently without concomitant tri-iodothyronine production. Although these studies are conflicting on several points, especially with regard to the properties of the catalysing factor involved in this reaction, they all have in common that CN^- brings about a diminution of the degradation of thyroxine. We, however, found that CN^- did not decrease the conversion (Table 4). The explanation for this phenomenon could be that rat liver microsomal fraction catalyses the deiodination of thyroxine in two different ways: one, a non-specific CN^- -sensitive deiodination leading to products other than tri-iodothyronine and a second, CN^- -insensitive deiodination specifically producing tri-iodothyronine. Green (1975) reached the same conclusion. Neither of these two activities is, however, identical with iodotyrosine deiodinase (Schwartz *et al.*, 1969; Rosenberg & Ahn, 1969; Haibach, 1971; Visser *et al.*, 1975).

Several coenzymes separately tested with the microsomal fraction did not result in an enhancement of tri-iodothyronine production (Table 5), although we observed a small, but significant, increase in converting activity by the addition of NAD^+ to the homogenate (Visser *et al.*, 1975). The reason for this remains unclear. Assuming that, besides tri-iodothyronine, I^- is the only product, the reaction under investigation is clearly a reduction: $\text{thyroxine} + 2H \rightarrow \text{tri-iodothyronine} + HI$. In our particular situation dithiothreitol might supply the reducing equivalents.

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References

- Chiraseveenuprapund, P., Buergi, U., Goswami, A. & Rosenberg, I. N. (1975) (*Abstr.*) *Program Int. Thyroid Conf. 7th, Boston*, p.75, Excerpta Medica, Amsterdam
- Dawber, N. A., Galton, V. A. & Ingbar, S. H. (1971) *Endocrinology* **88**, 144–148
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–716
- Docter, R., Visser, T. J., Stinis, J. T., van den Hout-Goemaat, N. L. & Hennemann, G. (1976) *Acta Endocrinol. (Copenhagen)* **81**, 82–95
- Galton, V. A. & Ingbar, S. H. (1963) *Endocrinology* **73**, 596–605
- Green, W. L. (1975) (*Abstr.*) *Program Int. Thyroid Conf. 7th Boston*, p.74, Excerpta Medica, Amsterdam
- Gross, J. & Pitt-Rivers, R. (1952) *Lancet* **i**, 439–441
- Gross, J. & Pitt-Rivers, R. (1954) *Recent Prog. Horm. Res.* **10**, 109–115
- Haibach, H. (1971) *Endocrinology* **88**, 918–923
- Hesch, R. D., Brunner, G. & Söling, H. D. (1975) *Clin. Chim. Acta* **59**, 209–213
- Klebanoff, S. J. & Green, W. L. (1973) *J. Clin. Invest.* **52**, 60–72
- Larson, F. C., Tomita, K. & Albright, E. C. (1955) *Endocrinology* **57**, 338–344
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Nakagawa, S. & Ruegamer, W. R. (1967) *Biochemistry* **6**, 1249–1261
- Oppenheimer, J. H., Schwartz, H. I., Dillman, W. & Surks, M. I. (1973) *Biochem. Biophys. Res. Commun.* **55**, 544–550
- Pitt-Rivers, R., Stanbury, J. B. & Rapp, B. (1955) *J. Clin. Endocrinol. Metab.* **15**, 616–620
- Rabinowitz, J. L. & Hercker, E. S. (1971) *Science* **173**, 1242–1243
- Rosenberg, I. N. & Ahn, C. S. (1969) *Endocrinology* **84**, 727–737
- Samuels, H. H. & Tsai, J. S. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3488–3492
- Schwartz, H. L., Kozyreff, V., Surks, M. I. & Oppenheimer, J. H. (1969) *Nature (London)* **221**, 1262–1263
- Schwartz, H. L., Surks, M. I. & Oppenheimer, J. H. (1971) *J. Clin. Invest.* **50**, 1124–1125
- Stanbury, J. B., Morris, M. L., Corrigan, H. J. & Lassiter, W. E. (1960) *Endocrinology* **67**, 353–362
- Sterling, K., Brenner, M. A. & Saldanha, V. F. (1973) *Science* **179**, 1000–1001
- Tata, J. R. (1960) *Biochem. J.* **77**, 214–226
- Visser, T. J., van der Does-Tobé, I., Docter, R. & Hennemann, G. (1975) *Biochem. J.* **150**, 489–493
- Woebber, K. A. & Ingbar, S. H. (1973) *J. Clin. Invest.* **52**, 1796–1803
- Wynn, J., Gibbs, R. & Royster, B. (1962) *J. Biol. Chem.* **237**, 1892–1897