Kinetics of Enzymic Reductive Deiodination of Iodothyronines

EFFECT OF pH

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5'-Deiodination of thyroxine (yielding 3,3',5-tri-iodothyronine; reaction I) and of 3,3',5'-tri-iodothyronine (yielding 3,3'-di-iodothyronine; reaction II) and 5-deiodination of thyroxine (yielding 3,3',5'-tri-iodothyronine; reaction III) and of 3,3',5-tri-iodothyronine (yielding 3,3'-di-iodothyronine; reaction IV) as catalysed by rat liver microsomal fraction were studied at pH 6.5, 7.2 and 8.0. It was found that: (1) the K_m of reaction I was relatively independent of pH (approx. $3 \mu\text{M}$), whereas V was highest at pH 6.5 (63 pmol of 3,3',5-tri-iodothyronine/min per mg of protein); (2) the K_m of reaction II was lowest at pH 6.5 (0.035 μM), but V was highest at pH 8.0 (829 pmol of 3,3'-di-iodothyronine/min per mg of protein); (3) thyroxine inhibited reaction II competitively; K_i values were identical at pH 6.5 and 8.0 (1 μM); (4) for both reactions III and IV K_m was lowest and V was highest at pH 8.0. The results are compatible with the view that reactions I and II are mediated by a single enzyme (iodothyronine 5'-deiodinase) and that reactions III and IV are catalysed by a second enzyme (iodothyronine 5-deiodinase).

Deiodination is the main pathway by which thyroxine is metabolized *in vivo* (Cavalieri & Rapoport, 1977; Burman, 1978). Studies *in vitro* have demonstrated that reductive deiodination of iodothyronines is enzymic in nature and involves thiol compounds. Reduced glutathione is probably the endogenous cofactor (Visser, 1978). The primary products in this cascade of reactions are the metabolically active form of the hormone 3,3',5-tri-iodothyronine (tri-iodothyronine) and the inactive isomer 3,3',5'-tri-iodothyronine (reverse tri-iodothyronine).

Both have been observed *in vitro* to be further degraded to 3,3'-di-iodothyronine (di-iodothyronine; see Scheme 1). Evidence has been presented which suggests that all possible intermediates in the sequential deiodination of thyroxine are indeed generated (Rudolph *et al.*, 1978; Sakurada *et al.*, 1978).

We suggested that two enzymes, i.e. iodothyronine 5(3)- and 5'(3')-deiodinase, are responsible for the entire deiodination of thyroxine (Visser, 1978). This was mainly based on the pH-dependence of the several reactions and on clinical findings. The concept

Scheme 1. Two pathways for the sequential deiodination of thyroxine to di-iodothyronine

is supported by the observations of competitive inhibition of reaction I by reverse tri-iodothyronine (Chopra, 1977; Hüfner *et al.*, 1977; Kaplan & Utiger, 1978) and of reaction II by thyroxine (Kaplan & Utiger, 1978). As both k_{cat} and K_m may vary with pH, one cannot adequately characterize the pH-dependence of an enzyme-catalysed reaction with measurements at a single substrate concentration. In the present study, therefore, we measured the effect of pH on V and K_m for each of the four deiodination reactions. Rat liver microsomal fraction was used as the source of the enzymes (Visser *et al.*, 1976). Evidence was obtained suggesting that reactions I and II are mediated by a single enzyme (iodothyronine 5'-deiodinase), although the interaction of reverse tri-iodothyronine with the enzyme is quite different from the reaction with thyroxine.

Experimental

Preparation of rat liver microsomal fraction in 25 mM-Tris/HCl/3 mM-EDTA/3 mM-dithiothreitol, pH 7.4 (Visser *et al.*, 1976), and analysis of deiodination (Visser *et al.*, 1978) were done essentially as described before. Reaction mixtures consisted of 0.066 M-sodium phosphate, 3 mM-EDTA, 3 mM-dithiothreitol, pH 6.5, 7.2 or 8.0, and substrate and microsomal fraction in the concentrations indicated. The final volume was 0.25 ml. Reaction was initiated by addition of microsomal fraction and incubation was carried out at 37°C. Reaction was stopped by addition of 1 ml of 1.25% of the detergent Brij 35 (Sigma Chemical Co., St. Louis, MO, U.S.A.) in 0.06 M-barbitone/0.15 M-NaCl/0.1% bovine serum albumin, pH 8.6, kept at 0°C. This concentration of Brij has been shown to destroy enzymic activity completely and afforded a 100% recovery of iodothyronines. The method circumvents the use of volatile ethanol (e.g. Visser *et al.*, 1978) for the preparation of the extracts. In control experiments microsomal fraction was added after the Brij solution.

The amounts of product generated and of substrate remaining were measured in 50 μ l of the extracts by means of specific radioimmunoassays (Visser *et al.*, 1978). Standards were prepared in 1% Brij in 0.06 M-barbitone/0.15 M-NaCl/0.1% bovine serum albumin, pH 8.6.

The amount of iodothyronine measured in the control experiment was subtracted from that produced in the complete reaction mixture. Both incubations and radioimmunoassays were performed in duplicate.

The protein content of the microsomal fraction was measured after solubilization of 0.1 M-NaOH, by using the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. The sources of the various materials used are given in a previous paper (Visser *et al.*, 1978). For the determination of

K_m and V values the straight lines of double-reciprocal plots were drawn by the least-squares method applied to unweighted means.

Results

Dependence of reaction rates on concentration of microsomal protein

Fig. 1 demonstrated that at pH 7.2 the production of tri-iodothyronine and di-iodothyronine from thyroxine and of di-iodothyronine from tri-iodothyronine was linear with the concentration of the microsomal fraction in the reaction mixture up to approx. 0.1 mg of protein/ml. Conversion of reverse tri-iodothyronine into di-iodothyronine reached a maximum at approx. 0.02 mg of protein/ml, owing to exhaustion of substrate under these conditions. The amount of reverse tri-iodothyronine degraded was equal to the amount of di-iodothyronine produced. Less than 10% of added thyroxine, tri-iodothyronine and di-iodothyronine (0.01 μ M) was degraded by up to 0.7 mg of microsomal protein/ml. Similar findings were obtained at pH 6.5 and 8.0. In further experiments 70 μ g of protein/ml was used in the study of reactions I, III and IV and 3.5 μ g of protein/ml for reaction II.

Reaction I: 5'-deiodination of thyroxine

Double-reciprocal plots of tri-iodothyronine production rate as a function of thyroxine concentration

Fig. 1. Accumulation of tri-iodothyronine (●) and of di-iodothyronine (○) from 1 μ M-thyroxine and of di-iodothyronine from 0.5 μ M-tri-iodothyronine (▲) and from 0.01 μ M-reverse tri-iodothyronine (△) as a function of microsomal protein concentration at pH 7.2

For experimental details see the text. Incubation time was 6 min.

at three pH values are shown in Fig. 2. Apparently, the reaction obeys ordinary Michaelis–Menten kinetics with K_m values of approx. $3\ \mu\text{M}$, being somewhat lower at pH 7.2 than at pH 6.5 and 8.0 (Table 1). The k_{cat} of the reaction is strongly influenced by pH, as illustrated by a 5-fold decrease in V if pH is increased from 6.5 to 8.0.

Reaction II: 5'-deiodination of reverse tri-iodothyronine

Fig. 3 shows the dependence of di-iodothyronine production on the concentration of reverse tri-iodothyronine at various pH values. Apparently, this reaction also follows Michaelis–Menten kinetics. Increasing the pH from 6.5 to 8.0 effected a 7-fold increase in K_m and a 2.5-fold increase in V (Table 1).

Reaction III: 5-deiodination of thyroxine

To obtain an accurate estimate of the rate of 5-deiodination of thyroxine the amount of reverse tri-

iodothyronine converted into di-iodothyronine has to be taken into account (see also Visser *et al.*, 1978). The implications of this procedure are illustrated in Figs. 4 and 5. At pH 6.5 no significant accumulation of reverse tri-iodothyronine was observed, whereas at pH 8.0 approximately equal amounts of reverse tri-iodothyronine and di-iodothyronine were found (Fig. 4). Production of di-iodothyronine by the pathway via tri-iodothyronine is negligible (see below and Visser *et al.*, 1978). If the sum of the concentrations of reverse tri-iodothyronine and di-iodothyronine was taken as a measure of 5-deiodination of thyroxine, this reaction was found to follow Michaelis–Menten kinetics (Fig. 5). Values for K_m were calculated to be approx. $2\text{--}3\ \mu\text{M}$, being highest at pH 6.5 (Table 1). The V of this reaction is highest at pH 8.0, being about twice the value at pH 6.5 (Table 1).

Fig. 2. Lineweaver–Burk plot of the conversion of thyroxine into tri-iodothyronine at pH 6.5 (●), 7.2 (○) and 8.0 (▲)

For experimental details see the text. Microsomal protein concentration was $0.07\ \text{mg/ml}$, and incubation time 12 min. Results are means $\pm$ S.D. of five experiments.

Fig. 3. Lineweaver–Burk plot of the conversion of reverse tri-iodothyronine into di-iodothyronine at pH 6.5 (●), 7.2 (○) and 8.0 (▲) measured with a specific radioimmunoassay for the latter

For experimental details see the text. Microsomal protein concentration was $3.5\ \mu\text{g/ml}$, and incubation time 12 min. Results are means $\pm$ S.E.M. of four experiments.

Table 1. Parameters of iodothyronine 5- and 5'-deiodinase-catalysed reactions in rat liver microsomal fraction
Mean values $\pm$ S.D. are given with the numbers of experiments in parentheses.

Reaction	pH	$K_m\ (\mu\text{M})$			$V\ (\text{pmol/min per mg of protein})$		
		6.5	7.2	8.0	6.5	7.2	8.0
5'-Deiodination of thyroxine (5)		2.9 ± 0.6	2.3 ± 0.5	3.0 ± 0.6	63 ± 25	30 ± 17	13 ± 7
reverse tri-iodothyronine (4)		0.035 ± 0.010	0.064 ± 0.008	0.23 ± 0.06	342 ± 169	559 ± 230	829 ± 425
5-Deiodination of thyroxine (3)		3.3 ± 1.3	1.9 ± 0.4	1.8 ± 0.3	13 ± 6	18 ± 5	24 ± 2
tri-iodothyronine (5)		7.8 ± 0.9	6.2 ± 0.2	4.7 ± 1.1	21 ± 9	36 ± 7	42 ± 19

Reaction IV: 5-deiodination of tri-iodothyronine

Conversion of tri-iodothyronine into di-iodothyronine was also found to be a saturable process (Fig. 6). The K_m for this reaction was larger than for the other reactions, and was highest at pH 6.5. V was

similar to that of reaction III, in both magnitude and dependence on pH.

Inhibition of 5'-deiodination of reverse tri-iodothyronine by thyroxine

The effect of thyroxine on the conversion of reverse tri-iodothyronine into di-iodothyronine was studied at pH 6.5 and 8.0. The results demonstrate that at both pH values thyroxine inhibits this reaction competitively (Fig. 7). From the change in apparent

Fig. 4. Di-iodothyronine (a) and reverse tri-iodothyronine (b) production rates as a function of thyroxine concentration at pH 6.5 (●), 7.2 (○) and 8.0 (▲)

For experimental details see the text. Microsomal protein concentration was 0.07 mg/ml, and incubation time 12 min. Results are means $\pm$ s.d. of three experiments.

Fig. 6. Lineweaver-Burk plot of the conversion of tri-iodothyronine into di-iodothyronine at pH 6.5 (●), 7.2 (○) and 8.0 (▲)

For experimental details see the text. Microsomal protein concentration was 0.07 mg/ml, and incubation time 12 min. Results are means $\pm$ s.d. of five experiments.

Fig. 5. Direct (a) and double-reciprocal (b) plot of the sum of di-iodothyronine and reverse tri-iodothyronine production rates as a function of thyroxine concentration at pH 6.5 (●), 7.2 (○) and 8.0 (▲)

For experimental details see the text and the legend to Fig. 4. Results are means $\pm$ s.d. of three experiments.

K_m for reverse tri-iodothyronine the K_i value for thyroxine was calculated to be approx. $1\mu\text{M}$ at both pH 6.5 and 8.0 (Table 2).

Discussion

Studies *in vitro* of the enzymic reductive deiodination of iodothyronines have yielded apparently

conflicting results for the optimum pH of these reactions. Several possibilities may explain these discrepancies. In rat liver homogenate or microsomal fraction, pH may affect the rate of deiodination indirectly by changing the availability of substrate, owing to alterations in binding to non-enzyme constituents of these preparations (Höffken *et al.*, 1977). To obtain a true evaluation of the effect of pH on reaction rates, all added substrate must be free to interact with the enzyme concerned. It is conceivable that the dependence of deiodinase activities on pH may also be influenced by the nature of the cofactor used, e.g. the pK of its thiol group. Enzymic deiodination of iodothyronines as a function of pH may further depend on the concentration of substrate tested, since K_m and k_{cat} may be affected independently. We therefore decided to study the variation in these parameters in a narrow pH-range comprising most reported optimum values. Under the conditions used in the present study, reaction rates were proportional to the microsomal protein concentration in the presence of dithiothreitol. Gross non-specific binding of substrate to the membranes is therefore excluded. If such binding did occur, then an increase in microsomal protein concentration would result not only in increased enzyme concentration but also in decreased substrate availability (Visser *et al.*, 1978). However, deviation from linearity was observed only above 0.1 mg of protein/ml.

Reaction I (5'-deiodination of thyroxine) is reported to be optimal at pH 6.0 (Höffken *et al.*, 1977) or pH 7.0 (Chopra, 1977). The K_m for this reaction is variously reported as $1.6\mu\text{M}$ (Hüfner *et al.*, 1977), $2.5\mu\text{M}$ (Chopra, 1977) and $7.7\mu\text{M}$ (Kaplan & Utiger, 1978). It is demonstrated (Table 1) that reaction velocity as a function of pH at sub-saturating concentrations of substrate is determined by k_{cat} .

For reaction II (conversion of reverse tri-iodothyronine into di-iodothyronine), reported values of K_m and optimum pH vary considerably. The data presented in Table 1 may at least in part explain these apparent discrepancies. It was found that both K_m and V were highly dependent on pH; K_m was lowest at pH 6.5 and V was highest at pH 8.0. Since the effect of pH on K_m is predominant, the rate of di-iodothyronine formation at low substrate concen-

Fig. 7. Lineweaver-Burk plot of the conversion of reverse tri-iodothyronine into di-iodothyronine at (a) pH 6.5 and (b) at pH 8.0 in the absence (●) and presence of (○) $2.5\mu\text{M}$ - or (■) $5\mu\text{M}$ -thyroxine

For experimental details see the text. Microsomal protein concentration was $3.5\mu\text{g/ml}$, and incubation time 15 min. Results are means $\pm$ s.d. of three experiments.

Table 2. Estimation of the K_i of the inhibition by thyroxine of the conversion of reverse tri-iodothyronine into di-iodothyronine. K_i values were estimated by using the equation: apparent $K_m = K_m (1 + [I]/K_i)$, where the apparent K_m is $-1/\text{intercept}$ on the abscissa in the Lineweaver-Burk plot (see Fig. 6) and $[I]$ the concentration of the inhibitor.

Concn. of thyroxine (μM)	pH	Apparent K_m of reverse tri-iodothyronine (μM)		K_i of thyroxine (μM)	
		...		6.5	8.0
—			0.025	—	—
2.5			0.080	1.1	1.1
5.0			0.13	1.1	1.0

trations as used previously (Visser *et al.*, 1978) should be highest in a slightly acidic medium, as indeed we have found. At relatively high concentrations of reverse tri-iodothyronine as used by Hüfner & Grussendorf (1978) and Chopra *et al.* (1978), reaction velocities would be expected to follow k_{cat} . Both groups of investigators reported, in accordance with this conclusion, that conditions were optimal at approx. pH 8.0. The magnitude of the K_m at pH 7.2, $0.064 \mu M$ (Table 1), is in excellent agreement with the value published by Chopra *et al.* (1978), i.e. $0.065 \mu M$. The value of this parameter at pH 7.5 as reported by Kaplan & Utiger (1978) is substantially lower (7.5 nM). This may be due to the differences in techniques, as these authors studied the disappearance of substrate on reaction of reverse tri-iodothyronine with whole rat liver homogenate. It should be emphasized, since the reaction follows a Ping Pong mechanism, that not only V but also K_m is a function of cofactor concentration (see below). This may also apply to the other reactions.

Little is known about the conversion of thyroxine into reverse tri-iodothyronine (reaction III). Hüfner *et al.* (1977) and Höffken *et al.* (1977) showed that accumulation of reverse tri-iodothyronine on incubation of thyroxine with rat liver homogenate or microsomal fraction was highest at pH 9–9.5. It has been demonstrated that this is mainly due to the fact that the high rate of reverse tri-iodothyronine removal (reaction II) occurs at a lower pH (Hüfner *et al.*, 1977; Visser *et al.*, 1978). If this reaction was taken into account, 5-deiodination of thyroxine was found to be optimal at pH 8.0 (Visser *et al.*, 1978), which has also been observed by Cavalieri *et al.* (1977). The latter investigators studied reaction III in rat liver cytosolic fraction. This preparation contains little enzyme activity, but studies involving its use are apparently not hampered by reverse tri-iodothyronine-degrading activity (Cavalieri *et al.*, 1977). The results in Table 1 are in agreement with the data of Cavalieri *et al.* (1977) and Visser *et al.* (1978), since K_m is lowest and V is highest at pH 8.0.

Formation of di-iodothyronine from tri-iodothyronine (reaction IV) has been shown to proceed maximally at pH 8.0 (Chopra *et al.*, 1978; Visser *et al.*, 1978). This appears to be due to the low K_m and high V at this pH (Table 1). The magnitude of the K_m at pH 7.2 as found in the present study, $6.2 \mu M$, is equal to the value published by Chopra *et al.* (1978), i.e. $6 \mu M$.

It has been suggested that the sequential deiodination of thyroxine is mediated by two enzymes, i.e. iodothyronine 5- and 5'-deiodinase (Visser, 1978). This hypothesis was based mainly on the finding that the reactions involving 5'-deiodination of thyroxine or reverse tri-iodothyronine showed a similar pH-dependence (Visser *et al.*, 1978) and on the competitive inhibition by reverse tri-iodothyronine of

reaction I (Chopra, 1977; Hüfner *et al.*, 1977; see also Kaplan & Utiger, 1978). More recent observations support this concept in that it has been shown that reaction II is inhibited competitively by thyroxine (Kaplan & Utiger, 1978). Clinical observations are in line with this hypothesis, since in several situations production of tri-iodothyronine and degradation of reverse tri-iodothyronine in peripheral tissues are simultaneously diminished (Schimmel & Utiger, 1977). We also showed that reactions involving 5-deiodination of thyroxine or tri-iodothyronine exhibit similar pH profiles (Visser *et al.*, 1978). This is confirmed in the present paper by the demonstration of a similar dependence on pH of both K_m and V of these reactions (Table 1).

The present study, however, reveals that the influence of pH on the kinetic parameters of the 5'-deiodination reaction is dependent on the substrate used. We therefore thought it of importance to study the mutual inhibition by the substrates of reactions I and II at pH 6.5 and at pH 8.0. Since reverse tri-iodothyronine is rapidly degraded under conditions where the deiodination of thyroxine is investigated, these studies were limited to the effect of thyroxine on the 5'-deiodination of reverse tri-iodothyronine. Despite the large variation in K_m for the latter, the K_i for thyroxine was identical at pH 6.5 and 8.0 (Table 2). In concordance with Kaplan & Utiger (1978), the value of the K_i for thyroxine was close to the K_m for this compound in reaction I. It is concluded, therefore, that 5'-deiodination of thyroxine as well as of reverse tri-iodothyronine is mediated by a single enzyme.

These results strongly suggest that the presence of an iodine substituent at C-5 interferes with the interaction of the substrate with the 5'-deiodinase. Not only is the magnitude of K_m greatly increased and that of V diminished, but the dependence on pH of these parameters is changed considerably.

Recent investigations point to a Ping Pong mechanism (Laidler & Bunting, 1974) for reaction II and possibly for the deiodination of iodothyronines in general. Such a mechanism is supported by the following observations. (1) A decrease in the concentration of cofactor results in a proportional decrease in K_m and V of reaction II (T. J. Visser, unpublished work). (2) Inhibition by thiouracil is uncompetitive with respect to substrate (Chopra *et al.*, 1978). (3) Inhibition by thiouracil is competitive with respect to cofactor (T. J. Visser, unpublished work). Thiouracil has been shown to react specifically with sulphenyl iodides, yielding mixed disulphides (Cunningham, 1964), and iodothyronine deiodinases contain essential thiol groups (Visser, 1978). Thus the reaction pathway may be regarded as a trans-iodination and the subsequent reduction of an iodo-enzyme complex (sulphenyl iodide) by cofactor. The magnitude of both V and K_m would be dependent on

which step in this pathway is rate-limiting (Laidler & Bunting, 1974). It is conceivable that in reaction I the rate-limiting step is different from that in reaction II. This may explain the difference in behaviour of both V and K_m of these reactions with respect to variations in pH.

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