Biochimica et Biophysica Acta, 613 (1980) 41—51

BBA 68967

SOLUBILIZATION AND PARTIAL CHARACTERIZATION OF RAT LIVER IODOTHYRONINE DEIODINASES

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(Received August 17th, 1979)

Key words: Iodothyronine deiodinase; Thyroid hormone metabolism; (Rat liver)

Summary

Rat liver cells contain iodothyronine deiodinating enzymes (iodothyronine-5 and 5′-deiodinases), which are associated with the endoplasmic reticulum. In the present study, the iodothyronine deiodinases have been solubilized from the microsomal fraction of rat liver with 1.0% cholate and 0.25% of the polyoxyethylene ether W-1. Cholate can be effectively removed from the cholate extract with a mixture of the polystyrene beads XAD-2 and XAD-7. However, after some time, aggregation of proteins occurred.

Cholate solubilized iodothyronine-5′-deiodinase has an apparent molecular weight of 65 000 and a Stokes radius of 36—37 Å. The sedimentation coefficient is 4.3 S in 0.4—0.6% cholate, 7.6 S in 0.05% W-1 ether and 12.8 S in the absence of detergent. The enzyme solubilized with W-1 ether has an apparent molecular weight of approx. 200 000 and a Stokes radius of 52—56 Å in 0.025% W-1 ether. In the latter extract, the sedimentation coefficient of the deiodinase is 4.3—5.2 S under different conditions.

On DEAE-Sepharose chromatography, 70% of the bound deiodinases eluted with 0.1 M NaCl. The purification of this fraction was only 2-fold. Covalent chromatography, using activated thiol-Sepharose, resulted in approximately 3-fold purification of the deiodinases solubilized with W-1 ether, whereas in case of the cholate extract, no purification at all was obtained. Glutathione-Sepharose affinity chromatography resulted in no enrichment of the deiodinases.

Introduction

The parenchymal cell of rat liver can deiodinate thyroxine to 3,3′-diiodothyronine via 3,3′,5-triiodothyronine and 3,3′,5′-triiodothyronine [1,2]. This deiodination pathway is composed of four reactions, which are catalyzed by

enzymes. Several reports favour the concept that these reactions are catalyzed by two enzymes, viz. an iodothyronine-5′-deiodinase, responsible for the 5′-deiodination of thyroxine and 3,3′,5′-triiodothyronine, and an iodothyronine-5-deiodinase, catalyzing the 5-deiodination of thyroxine and 3,3′,5-triiodothyronine [1,3—5]. Upon subcellular fractionation, all deiodinase activities were found to be associated with the endoplasmic reticulum of the rat liver cell [2,6] and, at the present, no separation of deiodinase activities has been obtained.

In the present study, we have solubilized the iodothyronine deiodinases from the microsomal fraction, determined some of their physico-chemical properties and made preliminary attempts at purification.

Materials and Methods

Materials. L-Thyroxine, L-3,3′,5-triiodothyronine, dithiothreitol, Triton X-405, cholate, deoxycholate, Brij 35, polyoxyethylene ether W-1 (W-1 ether), trypsin (bovine pancreas, crystallized twice) and trypsin inhibitor (soya bean) were obtained from Sigma. L-3,3′,5′-Triiodothyronine was obtained by courtesy of Dr. E. Scheiffele (Henning GmbH, Berlin). Triton X-100 and digitonin were purchased from B.D.H. Lubrol PX was from I.C.I. and taurocholate from Calbiochem. Sephadex G-25 (Fine), DEAE-Sepharose CL-6B, activated thiol-Sepharose 4B, Sepharose 6B, Sephacryl S-200 SF, Sephadex G-150, and the marker proteins ribonuclease A, chymotrypsinogen A, ovalbumin, albumin aldolase, catalase, ferritin and thyroglobulin were obtained from Pharmacia, Uppsala, Sweden. Glutathione-Sepharose was obtained by elution of an activated thiol-Sepharose column with dithiothreitol. The resins XAD-2 and XAD-7 were purchased from Serva, Heidelberg, F.R.G.

Assay of iodothyronine deiodinase activity. Iodothyronine deiodinase activity was determined by incubating 5 μM thyroxine, 2.5 μM 3,3′,5-triiodothyronine or 0.5 μM 3,3′,5′-triiodothyronine with appropriate dilutions of the fraction to be tested in the presence of 0.08 M sodium phosphate (pH 7.0) and 2.5 mM dithiothreitol in a total volume of 0.2 ml. After incubation at 37°C for 10 min with shaking, the reaction was stopped by addition of 0.8 ml 0.06 M barbitone buffer, (pH 8.6), 0.1% bovine serum albumin, 0.1% SDS. The amounts of iodothyronine produced were measured directly in 50 μl of the extract with specific radioimmunoassays [1]. For the determination of the conversion of thyroxine into 3,3′,5′-triiodothyronine, the amount of 3,3′,5′-triidothyronine degraded into 3,3′-diiodothyronine was taken into account.

Solubilization of enzymes. Microsomes were prepared as described previously [2]. Solubilization was carried out using various procedures, viz. solvent extraction, treatment with detergents, high salt concentrations, trypsin or EDTA. For solvent extraction, acetone [7] and *n*-butanol [8] were used. The detergents used were Triton X-100 and X-405, Lubrol, cholate, deoxycholate, taurocholate, Brij 35, digitonin and W-1 ether. W-1 ether is a 1—1.78 mixture of cetyl 10 ether (Brij 56) and cetyl 20 ether (Brij 58). Detergent (in Tris-HCl/dithiothreitol buffer, consisting of 10 mM Tris-HCl (pH 7.4), 3 mM dithiothreitol) and an equal volume of microsomal fraction (containing approx. 15 mg protein/ml) were mixed and incubated for 45 min at 0°C. Final detergent con-

centrations ranged between 0.05 and 1.3% (w/v). Deiodinase activity was determined in the suspension and, following centrifugation (1 h at 110 000 × *g*), in the supernatant and in the pellet which was suspended in Tris-HCl/dithiothreitol buffer. Salt extraction with 0.2—4.0 M KCl (final concentration) [9] and treatment with 3—50 mM EDTA were carried out similarly. Solubilization with trypsin was attempted by incubating approx. 6 mg microsomal protein with 25—100 μg trypsin in a total volume of 0.9 ml (in Tris-HCl/dithiothreitol buffer). After incubation at 20°C for 2.5 h, or at 37°C for 1 h, the reaction was stopped by the addition of a 3-fold excess of trypsin inhibitor. Determination of deiodinases was carried out as described above.

Protein determinations. Protein was estimated by a modification of the Bradford method [10] using bovine serum albumin as standard [11]. This method is not influenced by several laboratory reagents, especially by dithiothreitol, which is used throughout all procedures. In preparations containing detergent, a modification [12] of the method of Lowry et al. [13] was used.

Removal of cholate. Cholate was removed from solubilized microsomal proteins by using a mixture of the polystyrene beads XAD-2 and XAD-7 (4 : 1, w/w) [14]. The column (1.0 × 19.5 cm) was equilibrated and eluted with 15 mM Tris-HCl (pH 9.0), 3 mM dithiothreitol (flow rate, 18 ml/h). Cholate was determined by liquid scintillation counting of [^{14}C]cholic acid (New England Nuclear, Boston, MA) of which a tracer quantity had been added to 1% sodium cholate.

Gel filtration. Gel filtration was carried out at 8°C on columns of Sepharose 6B (1.5 × 26 cm), Sephacryl S-200 (1.6 × 30 cm) and Sephadex G-150 (1.5 × 25 cm). The elution buffer contained 0.05 M Tris-HCl (pH 7.2), 0.1 M NaCl, 2 mM dithiothreitol with or without detergent (flow rates, 14, 28 and 9.5 ml/h, respectively). The molecular weight and the Stokes radius, R_s, were calculated from the plot of K_{av} vs. log molecular weight and of $\sqrt{-\log(K_{av})}$ vs. R_s, respectively, using marker proteins from Pharmacia calibration kits.

Sucrose gradient centrifugation. Linear sucrose gradients (4—24%, w/w) contained 20 mM Tris-HCl (pH 7.4), 2 mM dithiothreitol and in some experiments 0.6% cholate or 0.05% W-1 ether. A Beckman SW41 rotor was used for 16 h at 5°C and 153 000 × *g*. The sedimentation coefficients were determined by comparison with several marker proteins.

Chromatography on DEAE-Sepharose. The microsomal proteins solubilized with W-1 ether (approx. 15 mg protein) were loaded on a colum (1.6 × 8 cm) of DEAE-Sepharose CL-6B, which had been equilibrated with 20 mM Tris-HCl (pH 8.0)/2 mM dithiothreitol. The column was then eluted (flow rate, 20 ml/h) with 35 ml buffer and, subsequently, with similar volumes of buffer containing 0.1, 0.2 and 0.4 M NaCl.

Chromatography on thiol-Sepharose 4B. Approximately 20 mg of solubilized microsomal protein was added to the top of a Sephadex G-25 column (1 × 12 cm), which had been equilibrated with 40 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA, 0.15 M NaCl. The fractions containing deiodinase activity were pooled and this solution was then added to the top of a column (0.7 × 9 cm) activated thiol-Sepharose 4B, which had been equilibrated with the Tris-HCl buffer. The column was washed with the same buffer (flow rate, 6 ml/h) until the absorbance at 277 nm of the effluent was approx. zero. Elution was achieved

with the equilibration buffer containing 8 mM dithiothreitol. Conditions for chromatography on glutathione-Sepharose were the same as for activated thiol-Sepharose.

Results

Solubilization

In the control experiment (treatment with Tris-HCl/dithiothreitol only) the supernatant contained less than 10% of deiodinase activity (Table I). Treatment with Lubrol, Triton X-100, Triton X-405, deoxycholate, KCl, EDTA, acetone, *n*-butanol or trypsin yielded a soluble preparation displaying less than 15% of the activity of untreated microsomes. Taurocholate, digitonin and Brij 35 were able to solubilize 20—40% of the deiodinases. Of these detergents Brij 35 inactivated the deiodinases most strongly. In Table I are summarized results obtained with the detergents cholate and W-1 ether, which were most successful in solubilizing microsomes.

As demonstrated in Table I, cholate and W-1 ether are most effective at a final concentration of 1.0 and 0.25% (w/v), respectively. However, the apparent yield of solubilized enzyme was low. It was found that cholate or W-1 ether, added directly to the assay, caused a concentration-dependent decrease in microsomal deiodinase activity (Fig. 1). Therefore, the low yield of enzymatic activity after solubilization is at least partly due to a direct inhibitory effect of the detergent. Indeed, when the solubilized microsomes were assayed in a suitable dilution (yielding 0.005—0.025% cholate and 0.0025% W-1 ether, final concentrations), most of the deleterious action of cholate and W-1 ether was prevented (see specific activities in Table II). The data in Fig. 1 suggest that the 5-deiodinase activity is more affected by cholate than the activity of 5′-deiodinase, whereas with W-1 ether the reverse appears to be the case. Table II summarizes the isolation and solubilization of the 5′- and 5-deiodinase. It can be seen that cholate decreases the specific activity of the deiodinases by approx. 30%, whereas the decrease in case of W-1 ether is less than 20%.

TABLE I

DEIODINASE ACTIVITY AFTER SOLUBILIZATION OF MICROSOMES

Conversion studies were done at pH 7.0 using thyroxine as the substrate. Values for the supernatant are indicated as a percent of those for the mixture of microsomes and detergent before centrifugation and represent the mean of three experiments.

Detergent	Protein (% of initial)	5′-Deiodinase activity (% of initial)	5-Deiodinase activity (% of initial)
—	24	8	8
0.25% Cholate	44	20	19
0.50% Cholate	66	58	52
1.00% Cholate	80	80	78
1.30% Cholate	86	82	81
0.10% W-1 ether	49	22	26
0.25% W-1 ether	68	78	81
0.50% W-1 ether	85	82	84
1.00% W-1 ether	90	84	86

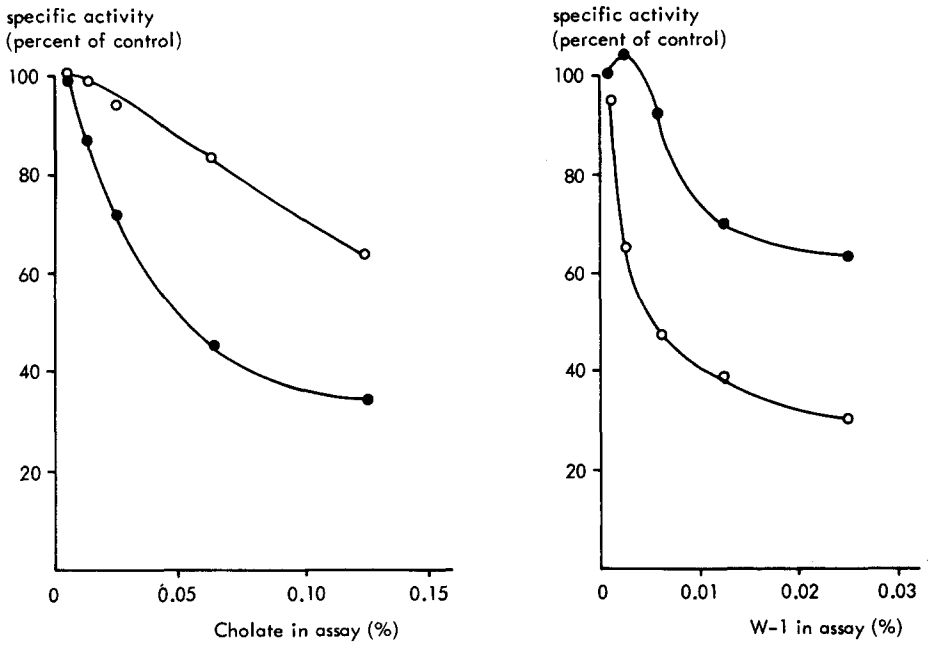

Fig. 1. Effects of cholate and W-1 ether on the specific activities of iodothyronine-5'- (○———○) and 5-deiodinase (●———●). Thyroxine was used as a substrate. Values are indicated as a percent of untreated microsomes.

Removal of cholate

Elution of microsomal proteins solubilized with 1.0% cholate on a mixture of the polystyrene beads XAD-2 and XAD-7 resulted in a more than 99% removal of cholate. Recovery of protein was approx. 95% and that of 5'-deiodinase activity (measured with 3,3',5'-triiodothyronine as the substrate) was 190%. This is probably due to stripping of cholate from the enzyme. In

TABLE II

ISOLATION AND SOLUBILIZATION OF RAT LIVER MICROSOMAL IODOTHYRONINE-5'- AND 5-DEIODINASE

The conversion studies were performed at pH 7.0, with thyroxine as the substrate.

Fraction	Total protein (mg)	Specific activity (pmol/mg protein per min)		Recovery (%)	
		5'-De-iodinase	5-De-iodinase	5'-De-iodinase	5-De-iodinase
Homogenate	1544	1.32	0.76	—	—
Microsomes	284	7.65	4.24	106	102
Supernatant fluid after solubilization with cholate	222	5.62	2.73	61	53
Supernatant fluid after solubilization with W-1 ether	173	6.23	3.63	53	55

this manner a crude soluble enzyme preparation was obtained essentially free of detergent. However, after some time aggregation occurred.

Gel filtration

Microsomal proteins solubilized with cholate or W-1 ether were applied to columns of Sepharose 6B, Sephacryl S-200 and Sephadex G-150. Apparent molecular weights and Stokes radii of the 5′-deiodinase after gel filtration on Sepharose 6B and Sephacryl S-200 are shown in Table III. In the absence of cholate in the solvent, the actual cholate concentration in the fractions containing deiodinase activity after chromatography on Sephacryl S-200 was approx. 0.05 mM (measured by using ^{14}C-labeled cholate). Apparently, this amount is sufficient to keep the protein in a soluble state. No cholate was measured in deiodinase containing fractions after Sepharose 6B chromatography. The results obtained with Sephadex G-150 are not included in this table, because the molecular weight of the deiodinase was, under all conditions, higher than 200 000 on this column. It is evident that the molecular weight and Stokes radius of the 5′-deiodinase are not only dependent on the solubilizing agent, but also on the type of gel filtration and the presence of detergent in the elution buffer. The apparent molecular weight and the Stokes radius of the 5′-deiodinase in the cholate extract are approx. 65 000 and 36—37 Å, respectively. For the W-1 ether extract, these values are 160 000—230 000 and 52—56 Å, respectively.

Sucrose gradient centrifugation

In Table IV, the sedimentation coefficients of the 5′-deiodinase under several conditions are shown. The deiodinase solubilized with W-1 ether had a sedimentation coefficient (4.3—5.2 S), which was almost independent of the presence of detergent in the gradient. However, in the cholate extract the enzyme had a high sedimentation coefficient (12.8 S) in the absence of any detergent in the gradient, pointing to aggregation. This also appeared to be the case after removal of cholate with the XAD-resin. In the presence of 0.05%

TABLE III

SEPHAROSE 6B AND SEPHACRYL S-200 GEL FILTRATION DATA OF MICROSOMAL 5′-DEIODINASE

The molecular weight and Stokes radius (R_S) of the 5′-deiodinase solubilized with cholate and W-1 ether are given. 3,3′,5′-Triiodothyronine was used as a substrate. n.d., not determined.

Sample	Sepharose 6B chromatography		Sephacryl S-200 chromatography	
	Molecular weight	R_S (Å)	Molecular weight	R_S (Å)
Cholate extract	>700 000	>100	60 000—70 000	34—37
Cholate extract (0.4% cholate in eluent)	60 000—70 000	36—39	65 000—75 000	35—38
W-1 Ether extract	160 000	53	>300 000	>60
W-1 Ether extract (0.025% W-1 ether in eluent)	n.d.	n.d.	200 000—230 000	52—56

TABLE IV

SEDIMENTATION COEFFICIENTS ($s_{20,w}$) OF SOLUBILIZED 5'-DEIODINASE

3,3',5'-Triidothyronine was used as substrate.

Sample	Detergent in gradient	$s_{20,w}$ ($\times 10^{-13}$)
Cholate extract	none	12.8
Cholate extract	0.6% cholate	4.3
Cholate extract	0.05% W-1 ether	7.6
Cholate extract after removal of cholate	none	12.8
W-1 Ether extract	none	4.3
W-1 Ether extract	0.6% cholate	5.2
W-1 Ether extract	0.05% W-1 ether	5.2

W-1 ether, the sedimentation coefficient was 7.6 S and in the presence of 0.6% cholate it was 4.3 S.

DEAE-Sepharose chromatography

The DEAE-Sepharose column bound approx. 80% of added 5'-deiodinase at pH 8.0 (Fig. 2). Nearly 70% of this bound enzyme could be eluted with 0.1 M NaCl. At concentrations higher than 0.2 M NaCl, no deiodinase activity was bound to the column, whereas protein was still present. The 5-deiodinase showed a similar profile. Recovery of total deiodinase activity was approx. 50% (protein recovery 100%) and enrichment of the enzyme in the fraction eluted with 0.1 M NaCl was only 2-fold.

Chromatography on thiol-Sepharose 4B

After removal of dithiothreitol from W-1 ether-solubilized microsomal proteins by Sephadex G-25, the sample was applied to activated thiol-

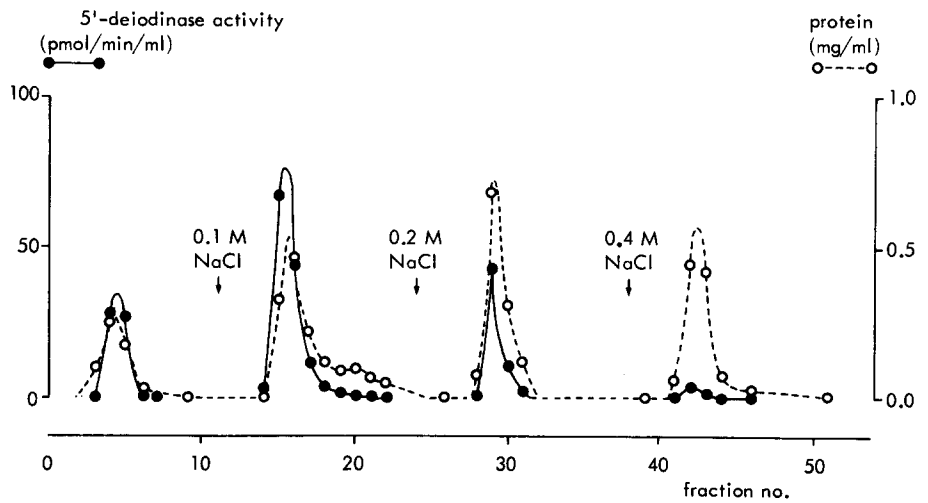

Fig. 2. DEAE-Sepharose chromatography, pH 8.0, of microsomes solubilized with W-1 ether. The column was developed with a discontinuous gradient of KCl in column buffer. 3,3',5'-Triiodothyronine was used as the substrate.

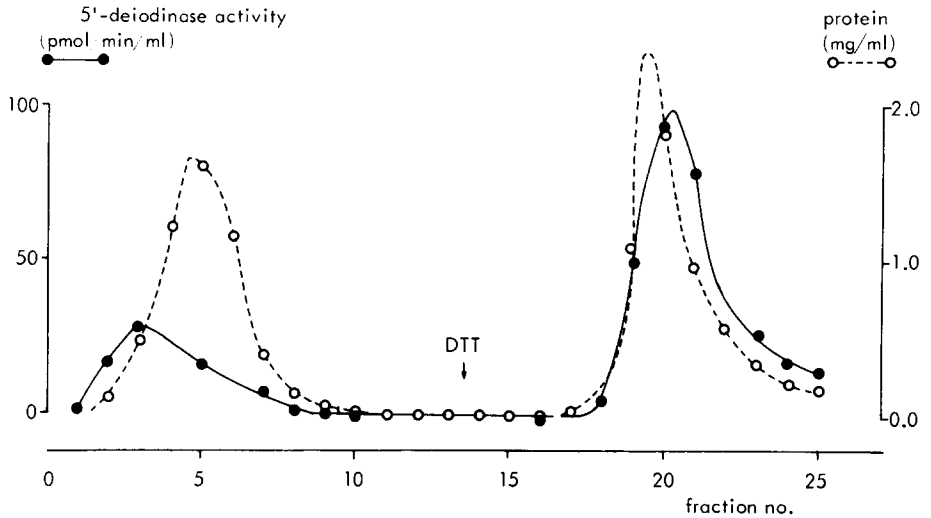

Fig. 3. Chromatography of W-1 ether-solubilized microsomes on thiol-Sepharose 4B. Elution of the bound enzyme was performed with buffer containing 8 mM dithiothreitol. 3,3',5'-Triiodothyronine was used as a substrate. DTT, dithiothreitol.

Sepharose. The elution pattern is seen in Fig. 3. About 25% of the deiodinase activity appeared in the column volume, whereas the remainder could be eluted with 8 mM dithiothreitol. However, many other proteins were bound to thiol-Sepharose too, resulting in little purification of the 5'-deiodinase (approx. 3-fold). 5-Deiodinase showed a similar profile. Protein and deiodinase recoveries were higher than 75%. After application of microsomes solubilized with cholate and prechromatographed on Sephadex G-25, about 90% of the deiodinase activity (together with approx. 75% protein) appeared in the column volume. The deiodinases were not adsorbed onto glutathione-Sepharose using microsomes solubilized with W-1 ether as well as with cholate.

Discussion

Effective solubilization of the microsomes was obtained with the detergents cholate and W-1 ether. The results with cholate are in agreement with those of Köhrle et al. [9] and Leonard and Rosenberg [15], although the latter studied rat kidney microsomes. In the presence of 1.0% cholate, 58 and 52% of the active form of iodothyronine-5'- and 5-deiodinase, respectively, could be solubilized. For 0.25% W-1 ether, these values are 50 and 54%, respectively. From our observations, it can be concluded that the thyroxine-deiodinating enzymes are integral (intrinsic) and not peripheral (extrinsic) membrane proteins because the enzymes cannot be dislodged from the membrane by the chelating agent EDTA nor by increasing the ionic strength (salt extraction) with KCl. However, Köhrle et al. [9] reported the solubilization of the deiodinase from microsomal fractions with 4 M KCl. We repeated the experiments and indeed we found deiodinating activity in the supernatant after centrifugation. But this turbid supernatant apparently had a similar density as the microsomes.

Upon dilution, all deiodinating activity could be pelleted. Therefore, no actual solubilization had occurred. Although solubilization does not result in separation of the deiodinating enzymes, the detergents cholate and W-1 ether affect the specific activity of iodothyronine-5′- and 5-deiodinase in a different manner (Fig. 1).

In many cases it is desirable to have a soluble enzyme preparation which is essentially free of detergent, especially in case of an ionic detergent. Therefore, we attempted to remove cholate from the soluble microsomal proteins. A mixture of the polystyrene beads XAD-2 and XAD-7 (4 : 1, w/w) appeared to be very successful for this purpose. However, after storage for a few days at 4°C, the proteins obtained in this manner aggregated visibly. Probably, aggregation occurred immediately after stripping of cholate from the proteins. In almost all chromatography procedures described in this paper, the applied cholate extract aggregated during elution, which was accompanied by considerable loss of activity of the deiodinases. The latter fact remained even in the presence of cholate during chromatography. For this reason, most column chromatography was carried out with microsomes solubilized with the non-ionic detergent, W-1 ether. Even in the absence of W-1 ether during chromatography, no observable aggregation occurred.

The apparent molecular weight of the 5′-deiodinase as determined by gel filtration turned out to be dependent on the presence and type of detergent and surprisingly, also on the gel filtration support. The results suggest that aggregation took place during chromatography of the cholate extract on the Sepharose 6B column in the absence of detergent in the eluent. Measurement of cholate in the deiodinase-containing fractions revealed that these were devoid of the detergent. On the other hand, some cholate was present in the deiodinase-containing fractions after chromatography on Sephacryl S-200, which, apparently, was sufficient to keep the deiodinase in a soluble form. Also, in case of the W-1 ether extract, differences in apparent molecular weights were observed to be dependent on the gel filtration support. Since we were not able to measure the amount of W-1 ether in the fractions containing deiodinase activity, we cannot conclude whether this was due to loss of detergent during chromatography.

The molecular weight of the deiodinase as determined by Sephadex G-150 chromatography was in all cases higher than 200 000. An explanation for this finding cannot be given at the moment. Nevertheless, from the gel filtration experiments on Sepharose 6B and Sephacryl S-200, it can be concluded that the apparent molecular weight of the 5′-deiodinase in the cholate extract is lower than in the W-1 ether extract. This may be explained by the fact that the deiodinase has more binding sites for W-1 ether than for cholate. Another possibility is that the enzyme binds to a whole micelle. In the latter case, the protein-detergent complex formed with W-1 ether is larger than that formed with cholate, because cholate has a micellar weight of only 900—1800, whereas for W-1 ether, having a similar structure as Lubrol and Brij, this value is higher than 50 000 [16].

The sedimentation coefficient of the 5′-deiodinase in the cholate extract in the presence of 0.6% cholate was 4.3 S. Similar values were obtained by centrifugation of the W-1 ether extract under various conditions. This value is

near the sedimentation coefficient of bovine serum albumin, which is 4.4. S. The approximate molecular weight of the enzyme is therefore similar to the one determined by gel filtration. Omission of cholate in the sucrose gradient resulted in aggregation, whereas exchange of cholate for a low concentration of W-1 ether, yielded partial aggregation of the deiodinase. The sedimentation coefficients of the 5′-deiodinase in the cholate and W-1 ether extract show only a slight difference, whereas their apparent molecular weights as determined by gel filtration show a much greater difference. This may be explained by their different hydrodynamic properties. Protein-W-1 ether complexes have a low sedimentation coefficient and a high Stokes radius, whereas protein-cholate complexes lack a similar combination [16].

DEAE-Sepharose chromatography of the W-1 ether-solubilized deiodinases at pH 8.0 yielded essentially three activity peaks. A minor peak eluted with buffer, a major peak with 0.1 M NaCl and a minor peak with 0.2 M NaCl. However, purification was very modest (approx. 2-fold). This is also true for chromatography on activated thiol-Sepharose. Since it is known that iodothyronine deiodinases contain essential sulfhydryl groups [17], it seemed worthwile trying to purify these enzymes by chromatography on thiol-Sepharose. It appeared that approx. 75% of the enzymes solubilized with W-1 ether, were absorbed onto this column. The bound deiodinases could be eluted from the column with the activating sulfhydryl reagent dithiothreitol [1,17]. However, many other proteins were bound to thiol-Sepharose too, resulting in very little purification of the deiodinases. The deiodinases were neither resolved by DEAE- nor by thiol-Sepharose. Unexpectedly, no binding occurred on a glutathione-Sepharose column, whereas glutathione is thought to be the endogenous cofactor of iodothyronine deiodinases [1,4].

In conclusion, iodothyronine deiodinases of the endoplasmic reticulum of rat liver cells can be solubilized with cholate and W-1 ether, and these enzymes are integral and not peripheral membrane proteins. The 5′-deiodinase has a molecular weight of about 65 000 in cholate and of approx. 200 000 in W-1 ether, a Stokes radius of 36—37 Å in cholate and of 52—56 Å in W-1 ether, a sedimentation coefficient of 4.3 S in cholate and 5.2 S in W-1 ether; it contains accessible sulfhydryl groups and has negligible affinity for glutathione under the conditions used. Up to now, no molecular evidence has been obtained for the existence of two deiodinating enzymes. This is not surprising, because the purification of 5′-deiodinase activity after column chromatography was only very modest.

Acknowledgements

This work was supported by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). Thanks are due to Dr. J.W.O. van den Berg for valuable advice and Mrs. C. Boot for typing the manuscript.

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