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PURIFICATION AND PARTIAL CHARACTERIZATION OF IODOTHYRONINE 5′-DEIODINASE FROM RAT LIVER MICROSOMES

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Summary: We have isolated and purified iodothyronine 5′-deiodinase from rat liver microsomes to homogeneity as judged by PAGE and analytical HPLC. The enzyme progressively lost activity after solubilization, and specific activity enhancement was a modest 22-fold, but the final preparation still had substantial activity and was used for molecular characterization. The enzyme had an M_r of 56,000 with a single band in SDS-PAGE, suggesting absence of subunit structure. The high K_m, and the GSH-responsive low K_m, activities were co-purified, but the low K_m enzyme lost GSH-responsiveness upon pretreatment with dithiothreitol (DTT) and urea. The enzyme was strongly inhibited by the iron chelator, α,α′-dipyridyl and showed a broad absorbance band at 410 nm. Spectral analysis with diethylpyrocarbonate (DEPC) revealed 5 histidine residues/mol enzyme, while enzyme activity was inhibited by DEPC in a pseudo-first order process with modification of 1 histidine residue/mol.

The conversion of thyroxine (T_4) to the active hormone, 3,5,3′-triiodothyronine (T_3) takes place principally in extrathyroidal tissues by the microsomal enzyme, iodothyronine 5′-deiodinase (I-5′D). Three variants of the enzyme have been described: a high K_m (~5 μM, type I) and a low K_m (~10 nM) enzyme occurring mainly in the liver and kidney (1-3), and a low K_m (~2 nM, type II) enzyme occurring in the brain, pituitary, and brown adipose tissue (4). Because the enzymes rapidly lose activity upon detergent-solubilization, they have proven difficult to purify, and their characterization has been based largely on studies with crude microsomal preparations. We report here the isolation from rat liver microsomes of an enzyme preparation which is homogeneous by polyacrylamide gel electrophoresis (PAGE) and analytical high pressure liquid chromatography (HPLC). Although the enhancement in specific activity in the final preparation was relatively modest, mainly because of rapid loss of enzymatic activity after solubilization and subsequent purification procedures, the purity and yield of the final preparation, which still retained substantial enzymatic activity, permitted a more precise study of the molecular characteristics of the hepatic high and low K_m enzymes. Our studies suggest that the type I (DTT-responsive) and low K_m (responsive to GSH as well as DTT) enzyme activities could actually be derived from the same enzyme protein. In addition, our studies reveal the presence of an active site histidine and a catalytically active iron center in the enzyme molecule.

MATERIALS AND METHODS

Chemicals. [3′ or 5′ -^{125}I]-T_4 and rT_3 (1250 μCi/μg) were purchased from Du Pont Co (Boston, MA). Nonradioactive rT_3 and CHAPS were from Behring Diagnostics (La Jolla, CA). Nonradioactive T_4, dithiothreitol, glutathione, urea, and hydroxylurea were from Sigma Chemical Co (St. Louis, MO). All other chemicals were of the highest purity commercially available.

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TABLE I
Summary of rat liver iodothyronine 5′-deiodinase purification

Step	Protein	Activity	Specific activity	Yield	Specific activity enhancement
	mg	*pmol h^{-1}*	*pmol h^{-1} mg^{-1}*	*%*	*-fold*
Microsomes	4,000	9,200	2.3	100	1.0
CHAPS extract	2,430	7,520	3.1	82	1.3
$(NH_4)_2SO_4$, 30-40% sat	1,400	6,730	4.8	73	2.1
DEAE-cellulose	56	700	12.5	7.3	5.4
Sephadex G-100	27	500	18.5	5.4	8.0
1st HPLC	13	415	31.7	4.5	13.8
2nd HPLC	2.6	135	52.2	1.5	22.7

[a]monitored with 0.5 nM rT_3 at 1 mM DTT.

Enzyme purification and assay. Liver microsomes from male rats (Charles River Laboratories, Wilmington, MA) were prepared by differential centrifugation in 0.25M sucrose, 1 mM EDTA, 10 mM Tris, pH 7.0. Enzyme activities at various stages of purification were monitored with 0.5 nM rT_3 as the substrate. In a typical purification experiment, approximately 2 gm of microsomal proteins were extracted with 40 ml of 10 mM Tris (pH 7.0), 1 mM EDTA, 6 mM CHAPS, 10% glycerol, by gently stirring at 4°C for 30 min and the supernatant recovered after centrifugation at 100,000g for 1h. The pellet obtained between 30-40% saturation of the supernatant with $(NH_4)_2SO_4$ was then dissolved in, and dialyzed against, the same solvent mixture, but containing only 0.1 mM CHAPS (TECG). The material was then applied to a DEAE-cellulose (Whatman DE52) column (5 X 5 cm) preeqilibrated with TECG, washed with 100 ml of TECG, and developed with a linear gradient of 0-0.5M NaCl. The activity peak was eluted at ~0.14M NaCl. The peak fractions were pooled, dialyzed against TECG, concentrated, and then applied to a Sephadex G-100 column equilibrated with the same buffer. The major peak emerged with an M_r of ~56,000 with a minor peak (M_r ~33,000) containing about 10% of the total activity. The fractions under the major peak were then pooled, concentrated, applied to a Waters ProteinPak 125 column, and subjected to HPLC, with TECG as the eluting solvent, at a flow rate of 1 ml/min. The fractions were monitored at 280 nm. Four peaks emerged containing 0, 0, 5, and 95% of total activity. The fractions under the major activity peak were then pooled, concentrated, and subjected to a second HPLC under the same conditions except that the buffer at this time did not contain EDTA (because EDTA interfered with the spectral characterization studies of the final preparation). This resulted in a single A_{280} peak containing all the I-5′-D activity. The fractions under the peak were pooled and this preparation (designated HP4 - the fourth peak in the 1st HPLC step) was used for further characterization studies. The results of the purification are summarized in Table I.

I-5′-D activity was assayed by quantitation of ^{125}I released from outer ring labeled T_4 or rT_3 by methods as described previously (5).

Electrophoresis. Polyacrylamide gel electrophoresis was performed on 0.75 mm slab gels (10%) using Tris-glycine buffer, pH 8.3 (Canalco) at 20 mA constant current. SDS-PAGE was performed on 15% gels, as described by Smith (6). Proteins were visualized by the silver stain procedure of Giulian *et al* (7).

RESULTS

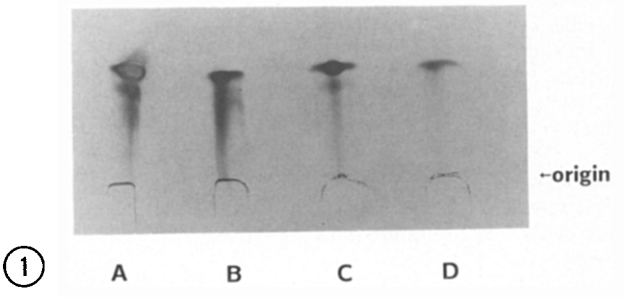
Characterization of the purified enzyme. Purified HP4 was homogeneous both in analytical HPLC and PAGE (see below). However, it showed only a modest enhancement of specific activity because of progressive loss of activity after detergent-solubilization. In absence of glycerol, the activity was completely lost during the DEAE-cellulose step. Inclusion of glycerol in the extraction medium and buffers thus provided some degree

of stabilization. While initial experiments gave only 5-10-fold enhancement in specific activity, as previously reported (8), modifications comprising rapid processing and narrower cuts in pooled peak fractions (peak tube ± σ) yielded preparations with about 23-fold increase in specific activity, as described below. Enzyme activity in HP4 was stable for about 3-4 weeks upon storage at -20°C.

Nondenaturing polyacrylamide gel electrophoresis of purified HP4 revealed a single stainable protein band (Fig. 1). The preparation also gave a symmetrical single peak in analytical HPLC (Fig. 2). Gel filtration of the purified preparation in Sephadex G-100 revealed a single zone containing all the enzymatic activity. The apparent molecular weight of the enzyme obtained from its elution behavior from a Sephadex G100 column calibrated with standard protein markers was ~56,000. Electrophoresis of the denatured enzyme on polyacrylamide gels containing sodium dodecyl sulfate also revealed a single band corresponding to a molecular weight of ~56,000, thus showing no evidence for subunit structures (Fig. 3).

Based primarily on immunological evidence, it has recently been reported that the microsomal enzyme, protein disulfide isomerase, has extensive sequence homologies with the 5′-deiodinase, and it has indeed been suggested that the two enzymes may be the same (9). Purified HP4, however, was inactive as a protein disulfide isomerase, as judged by the scrambled ribonuclease assay (10).

Spectral studies. (a) Absorption spectrum. The absorption spectrum of HP4 shows, besides the characteristic peak at 280 nm, a moderately strong absorbance band at 410 nm, with an A_{280}/A_{410} ratio of ~6 (Fig. 4). The exact nature of the chromophore responsible for this absorbance is currently uncertain. Treatment of the preparation with sodium dithionite or with reduced pyridine nucleotides had no effects on the intensity or the λ_{max} of this absorbance. The chromophore could be a heme group or an Fe-S center. In support of this, the enzyme was inhibited by the iron chelator, α,α′-dipyridyl, in a dose-dependent fashion (EC_{50} ~0.5 mM). However, the absence of any discernible band above 500 nm and the resistance of the 410 band to modification by reducing agents would argue against this possibility. The possible presence of a tyrosyl



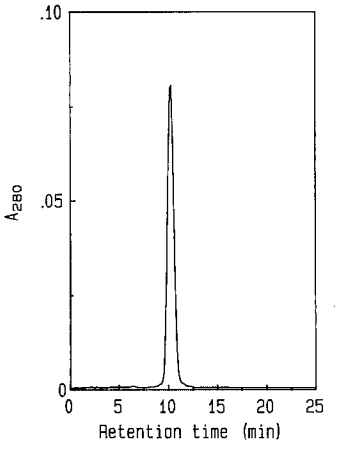
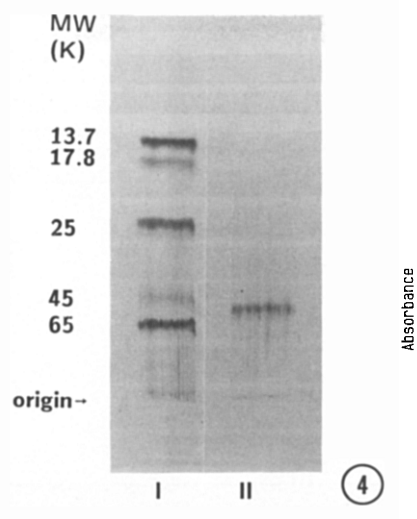


Fig. 1. Analysis of various steps in the purification of HP4 by polyacrylamide gel electrophoresis. Purification steps are described in 'Materials and Methods'. Samples of proteins from various steps (25 μg) were loaded on 10% gels and run for 4h at 20 mA. The proteins were visualized by silver staining. The samples were: A, DEAE-cellulose pool; B, after Sephadex G-100; C, 1st HPLC; D. 2nd HPLC.

Fig. 2. Analytical high pressure liquid chromatography of HP4. Purified HP4 (~75 μg) was injected into a Waters ProteinPak 125 column and developed at a flow rate of 1 ml/min with 10 mM Tris (pH 7.0), 1 mM EDTA, 0.1 mM CHAPS.



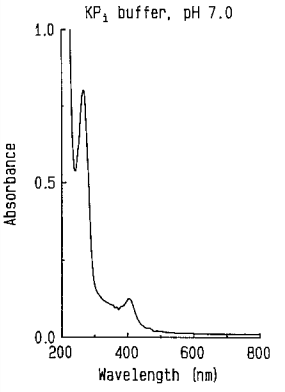
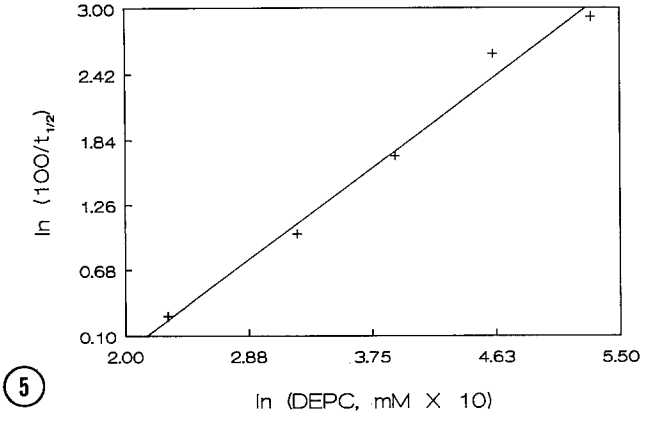


Fig. 3. Demonstration of purity of HP4 by SDS-PAGE. A 15% SDS-polyacrylamide gel was prepared and a mixture of molecular weight markers containing 10 μg of each component (slot 1) and 10 μg of HP4 (slot II) were applied. The markers were: ribonuclease (13.7 kDa), myoglobin (17.8 kDa), chymotrypsinogen A (25 kDa), ovalbumin (45 kDa), and BSA (65 kDa).

Fig. 4. Absorption spectrum of HP4. The sample (750 μg/ml) was in 0.1M potassium phosphate buffer, pH 7.0. The spectrum was recorded in a Cary 219 spectrophotometer in 0.5 ml cuvettes with 1 cm light path against a reference cuvette containing buffer alone.

free radical is also excluded because the band was unaffected by prolonged preincubation with high concentrations (upto 10 mM) of the free radical scavenger, hydroxylurea.

(b) Diethylpyrocarbonate (DEPC). In confirmation and further extension of the observations of Mol *et al* (11) with crude liver microsomal preparations, I-5´D activity of HP4 was strongly inhibited by DEPC, and this inhibition was diminished in presence of substrates and reversed by hydroxylamine. Also, as with microsomes (11), the inactivation followed a pseudo-first order process. Secondary plots of k_{app} *versus* DEPC



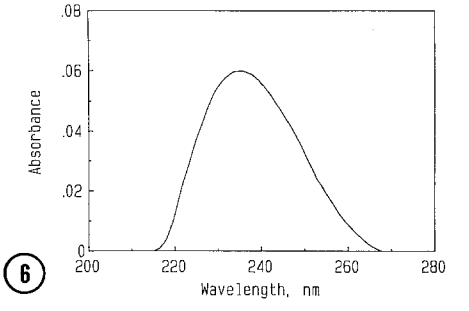


Fig. 5. Order of inactivation with respect to DEPC concentration. The linear plot gave a slope of 0.93.

Fig. 6. Difference spectrum of DEPC-modified HP4 minus native HP4. Baselines were first drawn with 2.5 mM DEPC in a Cary 219 spectrophotometer in 0.5 ml cuvettes with 1 cm light path against 0.1M potassium phosphate (pH 7.0) in the reference cell. Purified HP4 (100 μg) was then incubated with 2.5 mM DEPC for 10 min at 25°C in a total volume of 0.5 ml of the phosphate buffer in the cuvette and its spectrum recorded against 0.5 ml of buffer containing 100 μg unmodified HP4 in the reference cuvette.

TABLE II
Thiol responsiveness of HP4: activation by DTT and GSH

	Iodothyronine 5′-deiodinase activity (pmol I⁻/mg protein•h)	
Preincubation	DTT, 1 mM	GSH, 5 mM
I. Buffer alone		
T_4, 5 nM	45.4	30.3
rT_3, 0.5 nM	48.1	35.3
II. 10 mM DTT		
T_4, 5 nM	52.8	3.6
rT_3 0.5 nM	57.3	0
III. 5M urea		
T_4, 5 nM	39.3	8.8
rT_3, 0.5 nM	41.4	7.6

Purified HP4 (100 μg) was incubated at 37°C for 10 mins in 500 μl 0.1M K-phosphate (pH 7.0), 1 mM EDTA, either alone or containing 10 mM DTT. The samples were then dialyzed overnight against the buffer at 4°C with several changes of the dialyzing buffer. Aliquots of the enzyme preparations were then assayed for 5′-deiodinase activity with both DTT and GSH as thiol activators. The results are means of closely agreeing duplicates.

concentrations were linear (data not shown), indicating that the inactivation resulted from a bimolecular reaction between an enzyme residue with DEPC, and a second order inactivation rate constant of 0.007 min^{-1} was estimated from the slope. Plots of k_{app} *versus* [DEPC] were also linear (Fig. 5) with a slope of 0.93, thus indicating that inactivation involves modification of one histidine residue in the enzyme molecule (12).

Modification with DEPC. A difference spectrum of the DEPC-modified enzyme recorded against the native enzyme is shown in Fig. 6. The spectrum shows a broad maximum at 235-240 nm region and is consistent with the *N*-carboxyethylation of histidine residues in the enzyme protein by this reagent (13). There were no changes in A_{280}, thus arguing against any modification of tyrosine residues. Using a molar extinction coefficient of 3200 M^{-1} cm^{-1} for *N*-carbethoxyhistidine at 240 nm (14), the presence of 5 histidine residues per enzyme molecule was calculated.

Thiol responsiveness. At nanomolar substrate concentrations, purified HP4 responded to both DTT and low (physiological) concentrations of GSH as thiol activators. At 5 mM GSH, the K_m and V_{max} were ~10 nM and 58 pmol•mg $protein^{-1}$•h^{-1}, respectively, with T_4 as substrate. The corresponding values for rT_3 were ~5 nM and 75 pmol•mg $protein_{-1}$•h^{-1}, respectively. Thus, on the simplifying assumption that k_{cat} (the turnover constant) and K_m measure k_{+2} and K_s, respectively, the specificity constant, k_{cat}/K_m (15), of the enzyme for rT_3 is more than twice that for T_4, and therefore rT_3 appears to be the preferred substrate. The K_m value for T_4 in presence of 5 mM GSH reported here is about half that reported before for intact microsomal preparations (2), and probably reflects a modulating influence of microsomal lipids, which inhibit both HP4 and the microsomal enzyme in a competitive fashion[1], on enzyme conformation.

The parallelism in the behavior of the microsomal enzyme and the homogeneous enzyme preparation suggest that the DTT-dependent type I characteristics and the GSH-responsive low K_m characteristics may be

[1]A. Goswami and I.N. Rosenberg, manuscript in preparation.

attributed to the same enzyme protein under different thiol conditions. Furthermore, the GSH-responsiveness of HP4 at nanomolar substrate concentrations was completely lost after preincubation with DTT, followed by extensive dialysis to remove excess DTT, although DTT-responsiveness was retained, and even slightly enhanced (Table II). This suggests that the dithiol induced some structural alteration of the native enzyme, rendering it unresponsive to the naturally occurring monothiol, GSH. Similar inhibitory influences were also observed by exposing the enzyme to denaturing concentration of urea (5M) (Table II).

DISCUSSION

Because of the unavailability of sufficient amounts of the purified enzyme, characterization of the molecular forms and catalytic mechanisms of the I-5´Ds has been hampered. Several attempts (16-19) at purification of the enzymes have been unsuccessful because of the rapid loss of enzyme activity after detergent-solubilization. One report (18) describes a 2400-fold purification with the use of affinity chromatography on a PTU-sepharose column as the final step. However, the enzyme was only 50% pure as judged by PAGE, and the yield was very poor. Furthermore, in our hands, affinity chromatography on PTU-sepharose columns was completely ineffective, with no enhancement of specific activity with the introduction of this step.

The studies reported herein describe an enzyme preparation that is homogeneous in PAGE and analytical HPLC that still retains substantial amounts of enzyme activity (albeit with a somewhat modest increase in specific activity), and can be used for characterization studies. To our knowledge, this is the first report of the isolation of a homogeneous protein preparation showing I-5´D activity. Our procedure differed from the others in using 10% glycerol and 0.1 mM CHAPS throughout the purification procedure, and two consecutive preparative HPLCs as the final purification steps. The enzyme activity is unstable on storage and the relatively modest increase in specific activity after purification may have resulted from this loss of activity during processing.

Both gel filtration on Sephadex G-100 and SDS-PAGE showed the molecular mass of HP4 as 56 kDa, and no evidence for subunit structures. The enzyme thus differs from the type I enzyme in the LLC-PK1 cells (20), and the type II enzyme in cultured glial cells (21), where they were identified as consisting of a substrate-binding subunit bound to one or more additional subunits. The enzyme contains five histidine residues per molecule of which one appears to be essential for catalytic activity. The nature and functional role of the iron center, which may account for the absorbance peak at 410 nm, remains to be determined.

We have found that inclusion of DTT in the preparative medium, as has been customary in the preparative procedures reported from other laboratories (16-19), resulted in preparations which did not respond to GSH for enzymatic activity, although DTT-responsiveness was retained, or even improved. Similarly, preincubation of HP4 with DTT followed by extensive dialysis to remove excess DTT also resulted in loss of GSH-responsiveness with retention of DTT-responsiveness. This indicates the requirement for a 'locked-in' disulfide-bonded framework (22) for the GSH-responsiveness of the native enzyme. These observations, in conjunction with the findings that the low K_m (GSH-responsive) and the high K_m enzymes are co-purified in the homogeneous preparation, suggest strongly that the native enzyme may be the GSH-responsive low K_m enzyme and that the high K_m enzyme may be an artifact of DTT addition producing structural alterations in the native enzyme by disruption of intramolecular disufide bonds needed for the native enzyme conformation, and causing loss of GSH-responsiveness.

The reasons for the progressive loss of enzyme activity after detergent-solubilization are presently unclear. The greatest loss was in the DEAE-cellulose step after the $(NH_4)_2SO_4$ cut. It is possible that this loss

of activity is a consequence of removal from the microsomal lipid environment which may be needed for proper anchoring and alignment of functional groups (23). Reconstitution experiments using various cofactors and microsomal and other lipid preparations may help clarify this situation. Alternatively, the enzyme molecule may contain hydrophobic domains that are essential for an optimal folded conformation. Solubilization from the membranes may then be associated with alterations in hydrophobic free energy (24), with resultant destabilization of the folded structure. The stabilizing effect of glycerol on preservation of enzyme activity and the inhibitory effects of urea would support this conjecture.

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