Selective Affinity Labeling of a 27-kDa Integral Membrane Protein in Rat Liver and Kidney with N-Bromoacetyl Derivatives of L-Thyroxine and 3,5,3'-Triiodo-L-thyronine*

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Josef Körhle†§*, Ulla B. Rasmussen‡, Hartmut Rokos§, Jack L. Leonard§, and R. Dieter Hesch§

From the †Abteilung Klinische Endokrinologie, Medizinische Hochschule, D-3000 Hannover 61 and the ‡Forschungslabor Henning Berlin GmbH, D-1000 Berlin 42, Federal Republic of Germany, and the §Departments of Physiology and Nuclear Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01655

125I-Labeled N-bromoacetyl derivatives of L-thyroxine and 3,5,3'-triiodothyronine were used as alkylating affinity labels to identify rat liver and kidney microsomal membrane proteins which specifically bind thyroid hormones. Affinity label incorporation was analyzed by ethanol precipitation and individual affinity labeled proteins were identified by autoradiography after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Six to eight membrane proteins ranging in size from 17 to 84 kDa were affinity labeled by both bromoacetyl-L-thyroxine (BrAcT4) and bromoacetyl-L-triiodothyronine (BrAcT3). Affinity labeling was time- and temperature-dependent, and both reduced dithiols and detergents increased affinity labeling, predominantly in a 27-kDa protein(s). Up to 80% of the affinity label was associated with a 27-kDa protein (p27) under optimal conditions. Affinity labeling of p27 by 0.4 nM BrAc[125I]T4 was blocked by 0.1 μM of the alkylating ligands BrAcT4, BrAcT3, or 100 μM iodoacetate, by 10 μM concentrations of the non-alkylating, reversible ligands N-acetyl-L-thyroxine, 3,3',5'-triiodothyronine, 3,5-diiodosalicylate, and EMD 21388, a T3-antagonistic flavonoid. Neither 10 μM L-T3 nor 10 μM N-acetyltriiodothyronine or 10 μM L-triiodothyronine blocked affinity labeling of p27 or other affinity labeled bands. Affinity labeling of a 17-kDa band was partially inhibited by excess of the alkylating ligands BrAcT4, BrAcT3, and iodoacetate, but labeling of other minor bands was not blocked by excess of the competitors. BrAc[125I]T4 yielded higher affinity label incorporation than BrAc[125I]T3, although similar banding patterns were observed, except that BrAcT3 affinity labeled more intensely a 58,000 Da band in liver and a 53,000-55,000 Da band in kidney. The pattern of other affinity labeled proteins with p27 as the predominant band was similar in liver and kidney. Peptide mapping of affinity labeled p27 and p55 bands by chemical cleavage and protease fragmentation revealed no common bands excluding that p27 is a degradation product of p55. These data indicate that N-bromoacetyl derivatives of T3 and T4 affinity label a limited but similar constellation of membrane proteins with BrAcT4, incorporation greater than that of BrAcT3. One membrane protein (p27) of low abundance (2-5 pmol/mg microsomal protein) with a reactive sulfhydryl group is selectively labeled under conditions identical to those used to measure thyroid hormone 5'-deiodination. Only p27 showed differential affinity labeling in the presence of noncovalently bound inhibitors or substrates on 5'-deiodinase suggesting that p27 is likely to be a component of type I 5'-deiodinase in rat liver and kidney.

Many rat tissues bind and metabolize thyroid hormones by reductive mono-deiodination, conjugation, or radicalic, oxidative dehalogenation (1-3). Among these enzyme catalyzed reactions, the tissue-specific iodothyronine deiodinating isoforms play a key role in the bioactivation and inactivation of thyroid hormones (1). The isozymes of 5'-deiodinase (5'D) catalyze the production of metabolically active 3,3',5'-triiodothyronine (T3) from the main thyroid secretion product thyroxine (T4). 5-Deiodinase enzyme(s) catalyze the elimination of the metabolically active T3 (after sulfatation) and the generation of 3,3',5'-triiodothyronine (reverse T3, rT3) by the removal of a single tyrosyl-ring iodine. rT3 is devoid of thyroniunet action and also serves as a substrate for 5'D (4, 5). So far, these deiodinating isoforms have proved difficult to isolate and purify by conventional protein chemistry based on enrichment of enzyme activity, or by immunological procedures (1, 6-11). This is likely to be due to the lability, low abundance, and lipid dependence of these integral membrane proteins.

1 The abbreviations used are: 5'D, type I iodothyronine 5'-deiodinase; T4, 3,3',5'-triiodothyronine; T3, L-thyroxine; rT3, 3,3',5'-triiodothyronine; BrAcT4, N-bromoacetyl L-thyroxine; BrAcT3, N-bromoacetyl 3,3',5'-triiodothyronine; BrAc[125I]T4, N-bromoacetyl-[125I]-thyroxine; BrAc[125I]T3, BrAc[125I]3,3',5'-triiodothyronine; N-AcT4, N-acetyl-L-thyroxine; N-AcT3, N-acetyl-3,3',5'-triiodothyronine; EMD 21388, 4',6'-dimethoxy-3',5'-Br3,3'-CH-flavone; Hepes, 4(2-hydroxyethyl)-1-piperazinethanesulfonic acid; BSA, bovine serum albumin; DTE, diethyroxyethyl; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography.

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† To whom correspondence should be addressed: Abteilung Klinische Endokrinologie, Medizinische Hochschule, Postfach 610180, D-3000 Hannover 61, Federal Republic of Germany. Tel.: 090-49-511-5359-203. BITNET "koehrlegDHV-MMH1.bitnet."
The alkylation N-bromoacetyl derivatives of iodothyronines have been used as specific affinity labels to identify proteins that interact with thyroid hormones (12–20). Alternatively, UV-induced photolysis of \(^{131}I\)-labeled thyroid hormones which generates highly reactive radicals has also been employed to covalently label iodothyronine binding sites (20, 21). However, significant problems of specificity are often encountered with relatively long-lived and ill-defined iodothyronine radicals generated by UV-photolysis. Nonetheless, both approaches have proved useful in the characterization of thyroxine-binding globulin, transthyretin, the nuclear T3 receptor, mitochondrial proteins, and several thyroid hormone-binding-polypeptides in the plasma membrane and nuclear envelope. Sulphhydryl reagents such as iodoacetate or N-alkylmaleimides and the thioureylen 6-n-propyl-2-thiouracil irreversibly inactivate type I 5'-deiodinase by covalent modification of a highly reactive sulphhydryl group at or near the substrate binding site (3, 23). BrAcTg alkylation also inactivates rat liver microsomal type I 5'-deiodinase and enzyme substrates partially block labeling and protect the enzyme from inactivation (19, 35). We have previously shown that N-acyetilation or desamination markedly increased the affinity of the modified iodothyronine for type I 5'-D and transthyretin (24, 25). Both proteins also have higher affinities for T3 analogues with a 3'-monosubstituted phenolic ring compared to Tg analogues with a 3',5'-disubstituted phenolic ring (24, 25).

In this study, we used the enhanced avidity of N-substituted iodothyronines for the 5'-D substrate binding site and the ability of bromoacetyl derivatives to alkylate nucleophilic groups to label polypeptides in rat liver and kidney microsomes rich in 5'-D. Affinity labeling conditions were optimized and the specificity of affinity labeling was examined under nonequilibrium conditions. Our studies demonstrate the rapid and selective affinity labeling of a 27-KDa integral membrane protein in liver and kidney that contains a reactive sulphhydryl group in close proximity to a iodothyronine binding site preferring 3',5'-disubstituted iodothyronine analogues.

**MATERIALS AND METHODS**

**Reagents**—All reagents were of analytical or biochemical grade. Thyroid hormones were from Henning GmbH, Berlin, Federal Republic of Germany; purity of L-T3, L-T4, and L-T2 was >99.9%. EMD 21888, a T3-antagonistic flavonoid (29), was provided by Dr. K. Irmscher, E. Merck, Darmstadt, FRG.

**Synthesis of Affinity Labels**—BrAcTg, BrAcT2, and BrAcT3 were synthesized with minor modifications as described previously (15). 30 \(\mu\)l of \(N\)-hydroxysuccinimide bromoacetate (300 \(\mu\)l of a 0.1 M solution in 1,4-dimethoxyethane) was added to a suspension of 50 \(\mu\)l of 3,5,5;L-T3, or L-T2, and 80 \(\mu\)l triethylamine (80 \(\mu\)l of a 1 M solution in dimethoxyethane) in a mixture of 1 ml of dimethoxyethane and 0.5 ml of dimethylformamide. The reaction was carried out for 30–60 min at 40–45 °C during which the turbid solution clarified. Reaction products containing the BrAc-derivatized hormones were purified by thin layer (0.2 mm) chromatography on Silica Gel 60 (Merck, Darmstadt, FRG) with ethyl acetate/acetone acid (9/1, v/v) as a mobile phase. The prominent band at the appropriate RF (RF: BrAcTg, 0.50; BrAcT2, 0.55; BrAcT3, 0.60) was cut out, eluted with 2.5 ml of methanol, and quantified spectrophotometrically. The yield was between 50 and 70%. BrAc-derivatives were stored as methanol solutions in Teflon sealed glass vials in the dark at 20 °C.

**Synthesis of BrAcTg[131]I and BrAcTg[125]I—To 3 \(\mu\)g of BrAcTg or BrAcT3 in 5 \(\mu\)l of methanol were added 40 \(\mu\)l of 0.3 M phosphate buffer, pH 7.0, NaCl, 15 \(\mu\)g carrier-free –17 mCi/mg chloramine T (17 \(\mu\)l in 10 \(\mu\)l of H2O). After 60 s with vortexing, sodium (18 \(\mu\)l in 10 \(\mu\)l of H2O) was added. Products were either purified by TLC with methylene chloride/methanol/acetic acid (90/5/5, v/v) as mobile phase (Rf values: BrAcTg, 0.65; BrAcT3, 0.76; iodide, 0) or by reversed phase HPLC (Nucleosil RP18, 5 \(\mu\)m, Machery & Nagel, Duren, FRG, or Waters μBondapak C18 (0.46 x 25 cm) by gradient elution with acetone/trifluoroacetic acid at a flow rate of 1 ml/min. UV absorption was monitored at 254 nm with a LKB Ulvacord, radioactivity was measured on-line with a Berthold LB 505 radioactivity monitor, and data were analyzed by the Berthold HPLC chromatography system LB 510. Purity of affinity labels (specific activity >800 \(\mu\)Ci/\(\mu\)g) was >90%.

**Biochemical Assays**—In brief, liver homogenates (1/10, w/v) were prepared in ice-cold 250 mM sucrose by 4 strokes of a tightly fitting glass Teflon homogenizer. Liver microsomes and deoxycholate extracts were prepared as described previously (7, 25). Rat kidney cortex microsomes were prepared in kidney homogenization buffer (250 mM sucrose, 20 mM Hepes, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5), and taurodeoxycholate extracts (0.25%) were made essentially as described (6). Protein was determined by the method of Bradford (26) using bovine γ globulin standard.

**Affinity Labeling**—Microsomes or detergent extracts from individual rats (0.01–1 mg of protein/ml final volume) were affinity labeled in a total volume of 100 or 400 \(\mu\)l composed of 0.1 M Tris-HCl, pH 7.4, or 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 10 mM DTE or dithiothreitol, 0.025% W1 detergent, BrAc[125]I1 (12 \(\times\) 10^6 to 1 \(\times\) 10^6 cpm/ml; specific activity 6,450 cpm/fg), plus 5'-D substrates or inhibitors as indicated. Incubations were carried out for 20 min at 4, 25, or 37 °C. Affinity labeling was stopped by addition of 2–10 volumes of ice-cold ethanol and precipitated protein collected by centrifugation at 15,000 \(\times\) g for 10 min in a microcentrifuge. Identification of affinity labeled proteins was observed if affinity labeling reactions were stopped by addition of excess alkylation reagent (1 mM iodoacetate, final concentration) followed by ethanol precipitation or by adding 0.05% volume of 1% BSA in 0.15 M NaCl, for affinity labeling of rat kidney cortex microsomes.

Alternatively, aliquots (100 \(\mu\)l) of the affinity labeling reaction were mixed with 33 \(\mu\)l of 4 \(\times\) SDS-PAGE sample buffer (2% lithium dodecyl sulfate), 51 mM thioglycolic acid, 100 mM Tris-HCl buffer, pH 6.8, 7.5% glycerol (final concentrations) and denatured for 5 min at 95 °C (27).

**SDS-PAGE Analysis**—Affinity labeled proteins were separated by SDS-PAGE under reducing conditions using 10 or 11% gels (27) or exponential 10–20% gradient Laemmli gels (28). Thioglycolic acid (final concentration 3 mM) was added to the upper tank buffer to prevent aggregation of reduced proteins and to improve resolution of protein bands (27). Gels were stained with Coomassie Blue or silver as indicated (27), dried, and exposed to Kodak XAR-5 or DEF-2 film with Dupont-Cronex Lightning Plus intensifying screens at -70 °C. Quantitative analysis of distribution of radioactivity was done using a Berthold automatic Radio-TLC analyzer LB 2832 with a 18% SDS-PAGE gel and electrophoresis was performed as described above.

**CNBr Digestion and Proteolytic Fragmentation of p27 and p55**—Affinity labeled p27 and p55 bands were located in wet SDS-PAGE gels by the Berthold TLC scanner. 0.75 \(\mu\)m x 0.2 mm x 5–7 mm gel slices were cut out and either processed directly or electrophoreted in a Schleicher & Schuell biotrap at 50 V overnight in Laemmli buffer containing 100 \(\mu\)M thioglycolic acid. Partial CNBr cleavage of affinity labeled bands was performed directly in the gel slices after equilibration for 30 min at 4 °C in 0.5 M solution of 70% formic acid. Slices were transferred into 0.5 M of 1% CNBr in 70% formic acid, incubated at 25 °C for 2 h in the dark, and processed as described previously (27). Formic acid-susceptible Asp-Pro peptide bonds of p27 and p55 were cleaved by incubating gel slices with 70% formic acid at 65 °C for 5 h and processed as described above for the CNBr cleavage gel slices. After gel slices were positioned directly into the sample wells of a 18% SDS-PAGE gel and electrophoresis was performed as described above.

**Protease peptide mapping of electrophoresed, affinity labeled p27 and p55** (10,000–20,000 cpm) in solution was performed with trypsin, chymotrypsin, or Staphylococcus aureus V8 protease (50 \(\mu\)g/ml final concentration) in appropriate buffers for 10 h at 37 °C according to Cleveland et al. (41). Fragmentation was stopped by denaturing in...
Affinity Labeling of a 27-kDa Thyroid Hormone-binding Membrane Protein

RESULTS

Analysis of Covalent Affinity Label Incorporation—Iodothyronines bind noncovalently to many proteins presumably through hydrophobic interactions, and such binding confounds the analysis of covalent affinity labeling of membrane proteins by BrAc-derivatives of iodothyronines. Therefore, in preliminary experiments we examined several methods of separating covalently bound from noncovalently associated affinity label. Precipitation with ethanol alone or with acetone in combination with the addition of BSA as carrier protein or addition of 10,000-fold molar excess of the alkylating agent iodoacetate effectively terminated affinity label incorporation and allowed noncovalently bound affinity label to be quantitatively removed (data not shown) by collection of precipitated protein. Ethanol precipitation (2-10 volumes) of heat-denatured samples in SDS-PAGE sample buffer was also found to quantitatively precipitate affinity labeled proteins and allowed subsequent SDS-PAGE analysis of the affinity labeled proteins. This latter method was used in subsequent experiments because it avoids the complications by addition of BSA carrier protein used by Mol et al. (19) which is also found to quantitatively precipitate affinity labeled proteins in microsomal membrane preparations from liver and kidney. Bromoacetyl derivatives of T₃ and T₄ covalently labeled six to eight proteins present in microsomal preparations of liver (LM₃, A) or kidney (KM₄, B). In the absence of the competitive inhibitor EMD 21888 approximately 70–80% of the affinity label is found in a 27-kDa protein (p27). Addition of 10 µM EMD 21386, a flavonoid that competitively inhibits 5’D activity (2, 29), selectively blocked the affinity labeling of only p27 and paradoxically increased labeling of the 17-, 53-, and 58-kDa bands in LM₃ but not in KM₄. The other minor labeled bands were unaffected by EMD 21888 in LM₃ and KM₄. Densitometric analysis of Coomassie- or silver-stained lanes indicates that the prominent affinity labeled band at 27 kDa accounts for less than 0.5% of the total membrane proteins of deoxycholate-solubilized LM₃, (data not shown). BrAc[^125]T₃ affinity labeling of p27 was less intense than that observed with BrAc[^125]T₁, and no affinity labeling was observed by[^125]Iodide alone under these incubation conditions (Fig. 1B). As observed in ethanol precipitates of the affinity labeling reactions, no affinity labeling was observed if the reaction was immediately stopped or when the deoxycholate-solubilized microsomes were added after the stop reagents.

Time Course of Affinity Label Incorporation and Effects of Detergents and Reducing Agents—The time course of incor-
FIG. 2. Time course of total incorporation of BrAc\textsuperscript{125}I\textsubscript{T\textsubscript{3}} affinity label into deoxycholate-solubilized liver microsomes of normal rats and effects of iodothyronine analogues on affinity label incorporation. Deoxycholate (0.33%)-solubilized liver microsomes (10 μg/ml) were incubated as triplicates in 6-ml polypropylene tubes in a total volume of 3.2 ml of 100 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 10 mM DTE, 0.55% W1 at 25°C with 250,000 cpm/ml BrAc\textsuperscript{125}I\textsubscript{T\textsubscript{3}} (40 fmol/ml), in the absence (C) or presence of 10 μM diiodosalicylate (D) or 50 μM EMD 21388 (V). Reaction was started by addition of microsomes and 400-μl aliquots of the reaction mixture were removed at indicated time points, added to 150 μl of ice-cold 1% BSA in 150 mM NaCl, vortexed, and protein was immediately precipitated by 2 ml of ice-cold acetone. Affinity label incorporation was determined as protein-bound radioactivity after separation of supernatant and pellet by centrifugation at 3,000 × g. Data are presented as the mean of closely agreeing triplicates of one out of three similar experiments, after subtraction of the amount of radioactivity bound to BSA alone in the absence of microsomal extracts, typically in the order between 3 and 5% of total counts.

The progressive accumulation of alkylation of microsomal proteins by reversibly binding thyroid hormone analogues such as diiodosalicylate or EMD 21388 was greatest at short incubation times (<10 min) and showed a progressive decay of inhibition at longer incubation times. Identical results were obtained with BrAc\textsubscript{T\textsubscript{3}}, or if KM, or were used (data not shown). The progressive accumulation of alkylating affinity labels in proteins, even in the presence of a large excess of a competing ligand, is the expected consequence of the progressive nature of the irreversible interaction of the affinity label with a binding site(s) in dynamic equilibrium with reversibly binding ligands. This renders the systematic analysis of competition under equilibrium conditions impractical. Depicted in Fig. 3 are the effects of detergents and/or reducing agents on affinity labeling of liver microsomal proteins. Addition of DTE markedly increased BrAc\textsuperscript{125}I\textsubscript{T\textsubscript{3}} affinity label incorporation by 17% and resulted in the appearance of a prominent, new affinity labeled p27 band. Solubilization with deoxycholate, or the nonionic detergents Renex 690 or W1 ether further enhanced affinity labeling of the p27 polypeptide(s) by 7%. Affinity labeling of this p27 band was specifically blocked by BrAc\textsubscript{T\textsubscript{3}}, EMD 21388, and rT\textsubscript{3} (lanes 9–12), which showed little or no effect on the other labeled bands. We therefore included 0.025% W1 ether and reducing dithiols (10 mM DTE or dithiothreitol) in all subsequent affinity labeling reactions.

Comparison of the Effects of 5’D Substrates and Inhibitors on the BrAc\textsuperscript{125}I\textsubscript{T\textsubscript{3}} and BrAc\textsuperscript{125}I\textsubscript{T\textsubscript{3}}, Affinity Labeling Pattern in Liver and Kidney Microsomes—Shown in Fig. 4 is a comparison of the effects of 5’D substrates and inhibitors on affinity labeling of LM, with either BrAc\textsuperscript{125}I\textsubscript{T\textsubscript{3}} or BrAc\textsuperscript{125}I\textsubscript{T\textsubscript{3}}. The ligand-induced decreases in ethanol-precipitable affinity label incorporation into microsomal proteins (compare Table I) were due primarily to a loss of label from ~27, except with the alkylating agents iodoacetate and BrAc\textsubscript{T\textsubscript{3}}. These latter compounds also blocked affinity labeling of a 17-kDa band. Potent inhibitors of 5’-deiodination and the substrate, rT\textsubscript{3}, selectively decreased affinity labeling of p27 by 40–70% (compare Table I) and at the same time increased the label intensity in a 53-kDa doublet. EMD 21388 was an effective inhibitor of p27 affinity labeling, with little or no effect on other labeled bands. Nonradioactive BrAc\textsubscript{T\textsubscript{3}}, rT\textsubscript{3}, and the non-alkylating NA\textsubscript{T\textsubscript{3}}, are potent competitors for p27 labeling,
Affinity Labeling of a 27-kDa Thyroid Hormone-binding Membrane Protein

FIG. 4. SDS-PAGE analysis of specific affinity labeling of deoxycholate-solubilized rat liver microsomes by BrAc$^{[125]}$T$_3$ and BrAc$^{[125]}$T$_4$ and effect of competitors. Deoxycholate (0.17%)-solubilized rat liver microsomes (0.2 mg/ml) were incubated under standard conditions for 5 min at 25 °C with freshly HPLC-purified BrAc$^{[125]}$T$_3$ or BrAc$^{[125]}$T$_4$ (600,000 cpm/ml, 95 fmol) in a final volume of 120 μl. 40-μl aliquots were removed immediately after start of the reaction by addition of microsomes and after 5 min of incubation and added to 215 μl of ethanol or 10 μl of sample buffer. The competitors NAcT$_3$, T$_3$, NAcT$_4$, and rT$_3$ were present in 10 μM, the flavonoid EMD 21388 in 2 μM, and iodoacetate in 100 μM concentration. This autoradiogram (6 days exposed) illustrates the specific affinity labeling patterns of 3.2 pg of deoxycholate-solubilized microsomal radiolabeled proteins loaded per lane. Molecular mass markers: BSA (67 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa). Data are given for one out of three closely agreeing experiments using liver microsomes of individual rats. Table I gives the comparison of the quantitative evaluation of ethanol precipitation of affinity labeled proteins and the laser densitometric analysis of this autoradiogram.

Table I: Total and specific incorporation of BrAc$^{[125]}$T$_3$ and BrAc$^{[125]}$T$_4$ into deoxycholate-solubilized rat liver microsomes and competition by iodothyronine derivatives and iodoacetate.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Control</th>
<th>21388</th>
<th>T$_3$</th>
<th>rT$_3$</th>
<th>Iodoacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrAc$^{[125]}$T$_3$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibitor concentration (μM)</td>
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<td>2</td>
<td>10</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Total ethanol-precipitated counts (μM)</td>
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<td>39</td>
<td>64</td>
<td>36</td>
<td>22</td>
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<tr>
<td>Area counts 27 kDa/total (%)</td>
<td>83</td>
<td>32</td>
<td>80</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>BrAc$^{[125]}$T$_4$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total ethanol-precipitated counts (μM)</td>
<td>46</td>
<td>23</td>
<td>45</td>
<td>24</td>
<td>17</td>
</tr>
<tr>
<td>Area counts 27 kDa/total (%)</td>
<td>73</td>
<td>19</td>
<td>75</td>
<td>30</td>
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</table>

whereas T$_3$ shows no competition of affinity labeling of p27 under these reaction conditions. NAcT$_3$ shows no effect at these concentrations, similar to the lack of effect of T$_3$ (data not shown). While both affinity labels modify a similar constellation of microsomal membrane proteins, BrAcT$_3$ labeled more intensively the 17-, 30-, 33-, and 58-kDa proteins. However, BrAcT$_4$ gave consistently a 10-40% higher affinity label incorporation reflected by a more intense labeling of p27 compared to the T$_3$-derived affinity label under identical incubation conditions using affinity labels of the same specific radioactivity. Qualitative competition patterns for affinity labeling of p27 did not differ between the two affinity labels. These data suggest that the BrAc-iodothyronines react primarily with a reactive thiol group present in p27. Furthermore, iodothyronines with 3',5'-disubstitution (rT$_4$) and/or N-acetylation of the native alanine side chain (N-AcT$_4$) are better inhibitors of affinity label incorporation than T$_3$ and its derivatives. The comparable reaction and competition patterns of LM$_3$ and KM$_3$ with both affinity labels suggest that the major affinity labeled p27 protein(s) in liver and kidney microsomal fraction may be very similar if not identical.

Relationships between the 27-kDa and the 55-kDa Affinity Labeled Membrane Proteins—While p27 is prominently affinity labeled under incubation conditions identical to the S' D enzyme assay, and p27 affinity labeling is selectively enhanced
by reduced dithiols and blocked by 5'D substrates and inhibitors, previous estimates of the apparent molecular mass of the type I 5'D range from 50 to 60 kDa (1, 3, 6–8). To exclude the possibility that p27 was a degradation product of the other major affinity labeled protein(s), the 53–55-kDa polypeptide(s), we examined the peptide fragmentation patterns of these BrAc[125I]T4 affinity labeled proteins. Since peptide mapping is a fast and sensitive method for identifying and comparing individual proteins we isolated affinity labeled gel slices containing p27 and ~55 and compared their CNBr and formic acid chemical cleavage and protease fragmentation pattern. As shown in Figs. 5 and 6, no common radiolabeled fragments ranging between 26 and 5 kDa (lanes c and f) of p27 control, (lanes a and d) of liver (lanes a–c) and kidney (lanes d–f) are similar to each other (Fig. 5A, left panel). On the other hand, Fig. 5B (right panel) illustrates that, compared to p55 control (lanes i and j), the complex chemical cleavage patterns by CNBr (lanes h and k) and by formic acid (lanes g and l) of the p55 protein (lanes g–l) of liver (lanes j–l) and kidney (lanes g–i) are identical to each other but different from those of p27 (lanes a–f). This suggests a high similarity between the respective comparable affinity labeled proteins in rat liver and kidney. This finding is also supported by the completely different proteolytic cleavage pattern of p27 and p55 of rat kidney in solution (Fig. 6). No identical radiolabeled p27 and p55 fragments are obtained by partial digestion with three different proteases.

**Analysis of BrAcT3 and BrAcT4, Labeling of Microsomal Membrane Proteins in Brain, Skeletal Muscle, and Heart—** Incorporation for both thyroid hormone-derived BrAc-affinity labels under the standard incubation conditions is low or negligible in the 27-kDa protein(s) from microsomal fractions of rat liver and kidney. This finding is also supported by the completely different proteolytic cleavage pattern of p27 and p55 of rat kidney in solution (Fig. 6). No identical radiolabeled p27 and p55 fragments are obtained by partial digestion with three different proteases.

**DISCUSSION**

The integral membrane protein p27, affinity labeled by the BrAc-derivatives of T3 and T4, has a reactive sulphydryl group...
close to or directly at a binding site for rT3 or 3'5'-disubstituted T3 analogues. Selective and specific affinity labeling of p27 was demonstrated under incubation conditions which are almost identical to those for the 5'D enzyme assay. Affinity labeling of p27 was rapid and dependent upon temperature, protein, and presence of reduced thiols and detergents. p27, identified by SDS-PAGE, accounted for the majority of radioactively affinity label covalently bound to rat liver and kidney microsomes. Prolonged incubation and/or incubation in presence of excess BrAc-affinity label led to an increased labeling of several other proteins. However, no specific competition for the labeling of these proteins was observed by reversibly binding thyroid hormones and their analogues. This is likely to be due to the covalent binding characteristics of the affinity label, since alkylating BrAc-derivatives would be expected to react with nucleophiles (in all available thyroid hormone binding sites, both high and low affinity), given sufficient time and an excess of the affinity label, even in the presence of high-affinity reversible ligands.

Modification of the alanine side chain of naturally occurring thyroid hormones has been shown to affect the binding affinity for several serum- and cellular thyroid hormone-binding proteins such as transthyretin, the nuclear receptor, or type I 5'D (15, 24, 25, 32). Among several other thyroid hormone-binding proteins the latter have higher affinities for those thyroid hormone-derivatives where the positively charged amino function is either blocked (N-acetylation) or absent (carboxylic acid derivatives). This may explain, in part, the high affinity of BrAc-iodothyronines for p27.

The competition experiments for p27 affinity labeling revealed that most, if not all, of the reduction in affinity label incorporation was represented by changes in the affinity labeling of p27 in both liver and kidney microsomes and identified a p27 binding site specific for 3',5'-disubstituted iodothyronines. BrAcT3 was a more selective affinity label for p27 than BrAcT4. The flavonoid EMD 21388 which mimics the structure and functional groups of T4 (2, 26) was the most potent reversibly binding inhibitor for affinity label incorporation. In addition, the T3 analogue 3,5-diodosalicylate competed for affinity labeling, as did the 5'D inhibitor iopanoic acid (carboxylic acid derivatives). This may explain, in part, the high affinity of BrAc-iodothyronines for p27.

p27 protein(s) with the specific characteristics described above have not been identified with traditional affinity labeling protocols using either BrAc-derivatives or UV-induced photolysis of radiolabeled thyroid hormones in the absence of reducing dithiols and long labeling times (12-18, 20-22). However, several other (affinity) labeled proteins were described, which may be related to minor labeled bands observed in our experiments. Incorporation of radioactive affinity label into a 17-kDa band was slightly inhibited by excess cold BrAc-iodothyronine affinity labels and also by iodoacetate, but not by iodothyronines or by EMD 21388. Therefore, we suspect that this protein may have a reactive sulfhydryl residue but a low affinity for iodothyronines. We also routinely observed the affinity labeling of a doublet band in the 53-55-kDa range, which is more pronounced when labeling of p27 was blocked by competitors. However, no specific competition pattern was observed for these bands, which are abundantly represented in membranes and comprise up to 3% of the total protein per lane. Possibly these bands represent or contain as minor component the c-erb A T3-receptor (55 kDa) (15), or the membrane T3-binding protein(s) (p55, p58), recently identified as protein disulfide isomerase (16-18, 34).

Comparative peptide mapping, a fast and sensitive method for identifying and comparing individual proteins, clearly demonstrated that the two affinity labeled p27 and p55 proteins are not related to each other and that p27 is a proteolytic product of p55. Our studies indicate that both liver and kidney, rich in 5'D, contain a highly similar or even identical p27 protein, because both the chemical cleavage by CNBr or formic acid and the partial enzymic proteolysis reveal an identical pattern of radiolabeled fragments. However, also the partial peptide mapping patterns of p55, although different from those of p27, are identical in liver and kidney. An elegant study by Schoenmakers et al. (35), published after submission of this paper, using BrAcT3 affinity labeling of detergent or trypsin-treated liver and pancreas microsomes, rejects the hypothesis that protein disulfide isomerase, a protein with characteristics of the affinity labeled p55 band, is identical to 5'D and extends and supports our
Affinity Labeling of a 27-kDa Thyroid Hormone-binding Membrane Protein

findings, that p27 may represent a component of type I 5'D.

A minor affinity labeled band of 68 kDa, observed when we used BrAcT₂ as affinity label, has not yet been characterized and shows no specific competition pattern with any compounds tested to date. Control affinity labeling experiments using purified human thyroxine-binding globulin, human or rat transthyretin, or human or bovine serum albumin exclude the possibility that rat proteins corresponding to these proteins contribute to the labeling pattern obtained with microsomes (33, data not shown). Furthermore, also no labeling of the mitochondrial adenine nucleotide translocase, suggested to be identical to a 28-kDa mitochondrial T₂-binding protein (36) could be observed under our labeling conditions using rat liver and heart mitochondria (31). A cytosolic T₁- and T₂-binding protein in liver and kidney of 70 kDa determined by gel filtration was described by Sterling (37). However, the isolated microsomal membranes used here are essentially free of mitochondrial contaminants and cytosolic proteins were removed by the high-salt alkaline Tis-wash procedure of microsomal membranes (25). Some of the minorly labeled proteins may also represent iodothyronine-binding proteins (with lower affinity and reactivity for the BrAc-iodothyronines than the 27-kDa protein) identified by Dozin et al. (22) using the UV-induced direct photolabeling with T₁ and T₂. Recently, Robbins and coworkers (38-40) described a 28.3-kDa human serum T₁-binding protein, identified as apolipoprotein A1, which is also produced by the human cell line, Hep G2. However, no such protein has been identified in the rat.

The type I 5'-deiodinase isozyme of rat liver and kidney is the most likely candidate to contain the p27. Since the 5'D has a molecular mass of 50-60 kDa determined by gel filtration and sucrose density gradient centrifugation (6-8), this assumption is supported by our observations presented in this and the accompanying paper (43). (i) p27 is not related to a proteolytic product of p50 as shown by peptide mapping; (ii) iodocetate and 6-propyl-2-thiouracil, potent covalent 5'D enzyme inhibitors, prevent affinity label incorporation into p27; (iii) rats made hyper- or hypothyroid or starved rats (42) showed the expected alterations of 5'D activity and a parallel change in the quantity of p27; (iv) affinity labeling of microsomes inactivates 5'D activity; (v) p27 appears to represent a subunit of the 51-kDa 5'D holoenzyme in gel filtration; (vi) subcellular and tissue distribution of p27 and type I 5'D are almost identical; (vii) solubilized liver microsomes contain ~2-5 pmol of the p27 protein/per mg microsomal protein, which is <0.01% of the total microsomal or 0.001% of the total liver protein. This figure is in good agreement with previous estimates of the 5'D content in liver and kidney microsomes using radiolabeled iodocetate or 6-propyl-2-thiouracil as specific 5'D probes (3, 19, 23).

In summary, we have identified a p27 microsomal protein with a highly reactive thiol group close to or at a binding site for 3',5'-disubstituted iodothyronines, presumably representing the substrate binding subunit of 5'D. Affinity labeling of this p27 protein under conditions identical to those of type I 5'D catalysis provides a tool for the isolation and characterization of type I iodothyronine 5'-deiodinase, the enzyme catalyzing the bioactivation of T₁ to the thyromimetically active thyroid hormone T₂.

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Selective affinity labeling of a 27-kDa integral membrane protein in rat liver and kidney with N-bromoacetyl derivatives of L-thyroxine and 3,5,3'-triiodo-L-thyronine.

J Köhrle, U B Rasmussen, H Rokos, J L Leonard and R D Hesch


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