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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125I-Labeled N-bromoacetyl derivatives of L-thyroxine and 1,2,3-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-1,2,3-triiodothyronine (BrAcT3). Affinity labeling was time- and temperature-dependent, and both reduced disulfides 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]l-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 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 BrAcT3, BrAcT4, and iodoacetate, but labeling of other minor bands was not blocked by excess of the competitors. BrAc[125I]l-T4 yielded higher affinity label incorporation than BrAc[125I]l-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 T4 and T3 affinity label a limited but similar constellation of membrane proteins with BrAcT1, 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 isoforms of 5'-deiodinase (5'D) catalyze the production of metabolically active 3,3',5'-triiodothyronine (T3) from the main thyroidal 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 thyrocytiotic 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 1 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]l-T4, N-bromoacetyl-3,3',5'-triodothyronine; BrAc[125I]l-T3, N-bromoacetyl-3,3',5'-triiodothyronine; BrAc[125I]l-T4, N-acetyl-L-thyroxine; N-acetyl-L-thyroxine; BrAcT3, N-acetyl-3,3',5'-triiodothyronine; EMD 21388, 4',6-(OH)2,3',5'-Br2,3-CHR-flavone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; DTE, dithioerythritol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography.
The alkylating 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 125I-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.

Sulfhydryl reagents such as iodoacetate or N-alkylmaleimides and the thiourea 6-n-propyl-2-thiouracil irreversibly inactivate type I 5'-deiodinase by covalent modification of a highly reactive sulfhydryl group at or near the substrate binding site (3, 23). BrAcT₃ 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-acetylation 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 or desamination markedly increased the affinity of the modified iodothyronine for type I 5'D and transthyretin (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 sulfhydryl group in close proximity to a thyroxine 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-T₂, and L-T₄ was >99.9%. EMD 21388, a T₄-antagonistic flavonoid (29), was provided by Dr. K. Irmischer, E. Merck, Darmstadt, FRG.

Synthesis of Affinity Labels—BrAcT₁, BrAcT₂, and BrAcT₃ were synthesized with minor modifications as described previously (15). 30 μmol of N-hydroxyoxysuccinimide bromoacetate (300 μl of a 0.1 M solution in dichloromethane) was added to a suspension of 50 μmol of 5,5'-diiodothyronine and 0.3 ml of 50% acetic acid. The reaction was carried out for 30-60 min at 40-45 °C during which time the turbid solution clarified. Reaction products containing the BrAc-derivatized hor- mones were purified by thin layer chromatography on Silica Gel 60 (Merck, Darmstadt, FRG) with ethyl acetate/acetic acid (9:1, v/v) as a mobile phase. The band at the prominent position of Rₗ (BrAcT₁, 0.52; BrAcT₂, 0.65; BrAcT₃, 0.80) was cut out, eluted with two 0.5-ml aliquots 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 BrAc⁴¹IT₃ and BrAc⁵¹IT₃—To 5 μg of BrAcT₁ or BrAcT₂ in 5 μl of methanol were added 40 μl of 0.3 M phosphate buffer, pH 7.0, NaCl (carrier-free, -17 mCi/mg; 1 mCi in 10 μl) and chloramine T (1.5 mg in 10 μl of H₂O). After 60 s with vortexing, sulfite (18 μg in 10 μl of H₂O) was added. Products were either purified by TLC with methylene chloride/methanol/acetic acid (90:5:5, v/v) as mobile phase (Rₗ values: BrAcT₁, 0.26; BrAcT₂, 0.66; BrAcT₃, 0.76; iodide, 0) or by reverse phase HPLC (Nucleosil RP-18, 5 μm, Macherey & Nagel, Düren, FRG, or Waters µBondapak C₈ (0.46 × 25 cm) by gradient elution with octanethiol (25-95%) in 0.1% trifluo- roacetic acid in H₂O 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 >9000 μCi/μg) was >90%.

Biochemical Assays—In brief, liver homogenates (1/10, w/v) were prepared in ice-cold 250 mM sucrose solution by 4 strokes of a tightly fitting glass 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, 100 mM EDTA, 1 mM dithiothreitol, pH 7.5), and taurodoxocholate extracts (0.2%) 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 μl 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% Wt detergent, BrAc[IIT₁ (12 × 10⁻⁶ to 1 × 10⁻⁵ cpm/ml; specific activity 6,450 cpm/ fmol), plus 5' D substrates or inhibitors as indicated. Incubations were for 30-60 min at 4 °C. Affinity labeling conditions were optimized. After addition of 2-10 volumes of ice-cold ethanol and precipitated protein collected by centrifugation at 15,000 × g for 10 min in a microcentrifuge. Identical affinity label incorporation was observed if affinity labeling reactions were stopped by addition of excess alkylating reagent (1 mM iodoacetate, final concentration) followed by ethanol precipitation or by adding 0.07% volume of 1% BSA in 0.15 mM NaCl, followed by 5 volumes of ice-cold ethanol.

Alternatively, aliquots (100 μl) of the affinity labeling reaction were mixed with 33 μl of 4 × SDS-PAGE sample buffer (2% lithium dodecyl sulfate), 51 mM thiglycolic 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 Coomasie 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 0.35 × 10 cm counting chamber. The distribution of stainable protein was determined by laser densitometer (LKB Ultroscan 2202) with an automatic HP integrator. In addition, some gel lanes were cut into 2-mm slices and counted in a γ-counter. Molecular mass was determined by interpolation using BSA (68 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa) as standards, or prestained molecular mass markers (Diversified Biotech) or 5'-labeled marker proteins (Amersham Corp.).

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 mm × 0.2 mm × 5-mm gel slices were cut out and either processed directly or electrophoreted in a Schleicher & Schüll biorad at 50 V overnight in Laemmli buffer containing 100 μM thiglycolic acid. Partial CNBr cleavage of affinity labeled bands was performed directly in the gels slices after equilibration for 30 min at 4 °C in 0.5 M of 70% formic acid. Slices were transferred into 0.5 ml 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. Afterward, gel slices were positioned directly into the sample wells of a 18% SDS-PAGE gel and electrophoresis was performed as described above.

Protein peptide mapping of electrophoretically affinity labeled p27 and p55 (10,000-20,000 cpm) in solution was performed with trypsin, chymotrypsin, or Staphylococcus aureus V8 protease (50 μg/ml final concentration) in appropriate buffers for 10 h at 37 °C according to Cleveland et al. (41). Fragmentation was stopped by denaturing in

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RESULTS

Analysis of Covalent Affinity Label Incorporation—Iodothy-romones 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 affinity labeled by bromoacetyl derivatives of iodothyronines (33). No affinity labeling was observed if reactions were stopped immediately after addition of membranes or if stop reagents were added before membranes. Incubation of the affinity labeling reactions at 4 °C also resulted in little or no incorporation into microsomal proteins (data not shown).

Identification of BrAc[T3]T3 and BrAc[T3]T4-labeled Proteins in Liver and Kidney Microsomes—Shown in Fig. 1, A and B, are the patterns of BrAcT3- and BrAcT4-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 (LM, A) or kidney (KM, B). In the absence of the competitor EMD 21388 approximately 70–80% of the affinity label is found in a 27-kDa protein (p27). Addition of 10 μM EMD 21388, a flavonoid that competently 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 LM, but not in KM. The minor labeled bands were unaffected by EMD 21388 in LM and KM. 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 LM, (data not shown). BrAc[T3]T3 affinity labeling of p27 was less intense than that observed with BrAc[T3]T1, and no affinity labeling was observed by [125I]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-
corporation of BrAcT$_3$ into solubilized liver microsomes is shown in Fig. 2. BrAcT$_3$ incorporation into ethanol-precipitable protein increased rapidly over the first 2 min and slowed progressively thereafter. "Specific" affinity label incorporation, similar time course (data not shown). Competition for affinity defined as quantity of BrAc$[^{125}I]$T$_3$ blocked by the presence appearance of a prominent, new affinity labeled p27 band.

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 BrAcT$_3$, or if KM, were used (data not shown). The progressive accumulation of alkylation labeling in proteins, even in the presence of a large excess of a competing ligand, is the expected consequence of the processive nature of the reversible 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. Depleted 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$[^{125}I]$T$_3$ 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 BrAcT$_3$, EMD 21388, and rT$_3$ (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$[^{125}I]$T$_3$ and BrAc$[^{125}I]$T$_3$, 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$[^{125}I]$T$_3$ or BrAc$[^{125}I]$T$_3$. 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 alkylation agents iodoacetate and BrAcT$_3$. These latter compounds also blocked affinity labeling of a 17-kDa band. Potent inhibitors of 5'-deiodination and the substrate, rT$_3$, 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 BrAcT$_3$, rT$_3$, and the non-alkylating NAcT$_3$, 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[125I]T3 and BrAc[125I]T4 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[125I]T3 or BrAc[125I]T4 (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 NAcT3, T3, NAcT4, and rT3 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 μg 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[125I]T4 and BrAc[125I]T3 into deoxycholate-solubilized rat liver microsomes and competition by iodothyronine derivatives and iodoacetate

Total radiolabel incorporation was determined by ethanol precipitation of the reaction mixture. Laser densitometric scan of autoradiograms revealed the total and relative distribution of radioactivity between the 27-kDa band and other radiolabeled protein bands (expressed as percentage of total area counts of non-inhibited control): BrAc[125I]T4: total area = 1.27 × 10⁶, 27-kDa area = 1.05 × 10⁵; BrAc[125I]T3: total area = 0.81 × 10⁵, 27-kDa area = 0.59 × 10⁴. Data represent the quantitative evaluation of the experiment shown in Fig. 4.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>21388</th>
<th>T3</th>
<th>rT3</th>
<th>Iodoacetate</th>
</tr>
</thead>
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<tr>
<td>BrAc[125I]T3</td>
<td>Inhibitor concentration (μM)</td>
<td>0</td>
<td>2</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total ethanol-precipitated counts (% of total)</td>
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<td>39</td>
<td>64</td>
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<td>22</td>
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<tr>
<td>Area counts 27 kDa/total (%)</td>
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<td>80</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>BrAc[125I]T3</td>
<td>Total ethanol-precipitated counts (% of total)</td>
<td>46</td>
<td>23</td>
<td>45</td>
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<td>9</td>
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whereas T3 shows no competition of affinity labeling of p27 under these reaction conditions. NAcT3 shows no effect at these concentrations, similar to the lack of effect of T3 (data not shown). While both affinity labels modify a similar constellation of microsomal membrane proteins, BrAcT3 labeled more intensively the 17-, 30-, 33-, and 58-kDa proteins. However, BrAcT3 gave consistently a 10-40% higher affinity label incorporation reflected by a more intense labeling of p27 compared to the T3-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 (rT3) and/or N-acetylation of the native alanine side chain (N-AcT3) are better inhibitors of affinity label incorporation than T3 and its derivatives. The comparable reaction and competition patterns of LM and KM, 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 5'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[125]I, 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 peptides were unrelated. However, Fig. 5 shows that the CNBr-fragmentation map (26-, 24-, and 16-kDa fragments, lanes b and e) and formic acid cleavage pattern (several 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 brain, skeletal, or heart muscle (data not shown) and in mitochondrial fractions of rat liver and heart (31). These data suggest a rapid, tissue-selective, covalent affinity label incorporation of alkylating BrAc-derivatives of iodothyronines preferentially into one or several p27 protein(s) of liver and kidney microsomes. The reaction and competition characteristics suggest the involvement of reactive reduced sulphydryl group(s) during the alkylation reaction of the thyroid hormone binding site(s) of p27, which prefers rT3 and 3',5'-disubstituted T4 analogues (with N-acetylated side chain) compared to 3'-monosubstituted T4 analogues as ligands.

**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 radioactive 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 BrAcT3. The flavonoid EMD 21388 which mimics the structure and functional groups of T3 (2,26) was the most potent reversibly binding inhibitor for affinity label incorporation. In addition, the T3 analogue 3,5-diodosaliciclate competed for affinity labeling, as did the 5′D inhibitor iopanoic acid, a 2,4,6-triiodoaniline derivative (data not shown). N-AcT3, and rT3, were potent competitors for label incorporation, whereas 10 μM T3, N-AcT3, and T3 showed no competition in liver and kidney microsomes under these labeling conditions. Since higher T3 concentrations during short times of incubation also blocked p27 labeling (data not shown) the competition pattern seemed to be similar to the apparent "affinities" of thyroid hormones or their analogues for the type I 5′D.

rT3 has the lowest apparent Km and highest Vmax values in the 5′D reaction followed by NAcT3, T3, N-AcT3, and T3 (1, 3, 24, 25). The presence of reducing dithiols and our incubation conditions enable 5′-deiodination (1, 3) so that competing iodothyronine-5′D substrates such as rT3, N-AcT3, and T3 may be progressively deiodinated during prolonged affinity labeling. This could explain that the addition of reducing dithiols and detergent, which stimulate 5′D activity, alters the competition of p27 labeling by rT3 as shown in Fig. 3 and that 10 μM T3, in contrast to rT3, failed to compete for p27 labeling. On the other hand, no deiodination of the BrAc-iodothyronine affinity labels was observed during our short affinity labeling incubations (data not shown). Under identical incubation conditions we could partially block BrAcT3 affinity labeling of transthyretin by T3 and EMD 21388 but not by rT3 (33). This is compatible with the higher affinity of T3 compared to rT3 for the T3-binding protein transthyretin which does not metabolize iodothyronine substrates in contrast to 5′D. Our findings confirm and extend the data of Visser's group (19, 35) who showed that iodothyronines competed for incorporation of BrAcT3 into liver microsomes and block the BrAcT3 inactivation of the type I 5′D.

A second factor contributing to the rapid and selective affinity labeling of p27 may be the high reactivity of this alkylating thyroid hormone derivative with a specific sulfhydryl group in close proximity to the thyroid hormone binding site. This assumption is supported by several findings. Reduced dithiols increase specific affinity label incorporation, especially in presence of detergent, either by reducing essential sulfhydryl groups of the protein or by altering access to the thyroid hormone binding site. In addition, alkylating halocarbons, such as iodoacetate, block p27 affinity labeling in a dose-dependent manner. BrAc-iodothyronine affinity label incorporation was also markedly reduced in liver microsomal membranes prepared from 6-propyl-2-thiouracil-pretreated rats (43). 6-Propyl-2-thiouracil is known to form mixed disulfides with oxidized sulfhydryl groups at or near hydrophobic pockets. Both iodoacetate and thiouracil derivatives have been used to label and quantitate thyroid hormone binding sites and to inactivate type I 5′-deiodinase (3, 23). These data, together with the evaluation of structure-metabolism relationships of thyroid hormone analogues (24, 25), suggested the involvement of a highly reactive essential cysteine in the active site of type I 5′D, at or near the hydrophobic thyroid hormone binding pocket.

p27 protein(s) with the specific characteristics described here 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 (12–20–22). 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 55–55-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 not 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 revealed 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...
findings, that p27 may represent a component of type I 5'D. A minor affinity labeled band of 68 kDa, observed when we used BrAcT_2 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 T_3-binding protein (36) could be observed under our labeling conditions using rat liver and heart mitochondria (31). A cytosolic T_3- and T_2-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 microsomal contaminants and cytosolic proteins were removed by the high-salt alkaline Tris-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-iiodothyronines than the 27-kDa protein) identified by Dozin et al. (22) using the UV-induced direct photolabeling with T_3_ and T_2_. Recently, Robbins and coworkers (38-40) described a 28.3-kDa human serum T_3-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 ~27. Since the 5'D has a molecular mass of 50-60 kDa determined by gel filtration and sucrose density gradient centrifugation (6-8), this would imply that p27 is a subunit, specifically the substrate binding subunit, of the 5'D and contains the highly reactive essential reduced cysteine shown to participate in the catalytic cycle. 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) iodoacetate and 6-propyl-2-thiouracil, potent cofactor 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 ~27; (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 iodoacetate 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 iodothyroines, 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 T_3, to the thyromimetically active thyroid hormone T_3.

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Affinity Labeling of a 27-kDa Thyroid Hormone-binding Membrane Protein

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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