Photolabeling of Prostaglandin Endoperoxide H Synthase-1 with 3-Trifluoro-3-(m-[\textsuperscript{125}I]iodophenyl)diazirine as a Probe of Membrane Association and the Cyclooxygenase Active Site\* 

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Previous studies of the crystal structure of the ovine prostaglandin endoperoxide H synthase-1 (PGHS-1)/5-flurbiprofen complex (Picot, D., Loll, P. J., and Garavito, R. M. (1994) Nature 367, 243-249) suggest that the enzyme is associated with membranes through a series of four amphipathic helices located between residues 70 and 117. We have used the photoactivatable, hydrophobic reagent 3-trifluoro-3-(m-[\textsuperscript{125}I]iodophenyl)diazirine ([\textsuperscript{125}I]TID) which partitions into membranes and other hydrophobic domains to determine which domains of the protein interact with membranes. After photolabeling with [\textsuperscript{125}I]TID, ovine PGHS-1 was one of the major photolabeled products. Proteolytic cleavage of labeled PGHS-1 at Arg\textsuperscript{277} with trypsin established that [\textsuperscript{125}I]TID was incorporated into both the 33-kDa tryptic peptide containing the amino terminus and the 38-kDa tryptic peptide containing the carboxyl terminus. This pattern of photolabeling was not affected by the presence of 20 mM glutathione, indicating that the photolabeling observed for PGHS-1 was not due to the presence of [\textsuperscript{125}I]TID in the aqueous phase. However, nonradioactive TID as well as two inhibitors, ibuprofen and sulindac sulfide, which bind the cyclooxygenase active site of PGHS-1, prevented the labeling of the 38-kDa carboxyl-terminal tryptic peptide. These results suggest that [\textsuperscript{125}I]TID can label both the cyclooxygenase active site in the tryptic 33-kDa fragment and a membrane binding domain located in the 33-kDa fragment. Cleavage of photolabeled PGHS-1 with endoproteinase Lys-C yielded a peptide containing residues 25-166 which was labeled with [\textsuperscript{125}I]TID. This peptide contains the putative membrane binding domain of ovine PGHS-1. Our results provide biochemical support for the concept developed from the crystal structure that PGHS-1 binds to membranes via four amphipathic helices located near the NH\textsubscript{2} terminus of the protein.

The prostaglandin endoperoxide H synthase (PGHS)\textsuperscript{3}

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The abbreviations used are: PGHS, prostaglandin endoperoxide H synthase; TID, 3-trifluoro-3-(m-iodophenyl)diazirine; PG, prostaglandin; NSAID, nonsteroidal anti-inflammatory drug; ER, endoplasmic reticulum; ECL, enhanced chemiluminescence; PAGE, polyacrylamide gel electrophoresis.

\textsuperscript{3} The numbering of amino acids begins at the methionine at the translational start site of ovine PGHS-1.
out fully traversing it. Should this prediction hold true, PGHS-1 will be the first monotopic membrane protein for which the structure has been determined. However, there is currently no biochemical evidence supporting this prediction.

3-Trifluoro-3-[(m-125I)iodophenyl)diazirine ([125I]TID) is a hydrophobic, photoactivatable reagent which partitions into lipid bilayers making this reagent a useful tool for determining regions of proteins which are membrane-associated (22–24). In order to identify regions of PGHS-1 which are associated with the lipid bilayer, we examined the photolabeling by [125I]TID of PGHS-1 present in microsomal membranes prepared from ovine seminal vesicles. Our results indicate that [125I]TID photolabels a region of PGHS-1 which contains the putative membrane binding domain. We also found that [125I]TID can photolabel the cyclooxygenase active site of PGHS-1.

EXPERIMENTAL PROCEDURES

Materials—[125I]TID and ECL Western blotting reagents were from Amersham Corp. Protein A-Sepharcl 4B, ibuprofen, TWEEN 20, trypsin, chicken egg white trypsin inhibitor, and reduced glutathione were from Sigma. Endoproteinase Lys-C was from Boehringer Mannheim. Goat anti-rabbit IgG-horseradish peroxidase was from Bio-Rad. Frozen ovine vesicular glands were obtained from Oxford Biomedical Research. Nonradioactive TID was the generous gift of Dr. Jonathan Cohen (Harvard Medical School). Sulfinic sulfide was a gift from Merck.

Preparation of Microsomes from Ovine Vesicular Glands—Frozen ovine vesicular glands were sliced into thin strips with a razor blade and homogenized with a Polytron homogenizer in a HEPES buffer consisting of 20 mM HEPES, 20 mM glutamic acid, 2 mM magnesium acetate, and 200 mM sucrose, pH 7.5. The homogenate was centrifuged at 10,000 × g for 10 min to remove cell debris and mitochondria. The resulting supernatant was centrifuged at 200,000 × g for 1 h (3), and the microsomal pellet was resuspended in HEPES buffer by homogenization with a Dounce homogenizer. Ovine vesicular glands were sliced into thin strips with a razor blade and were irradiated at a distance of 5–10 cm with a hand held UV lamp for 10 min. Solubilized PGHS-1 was immunoprecipitated using the procedure described above.

Trypsin Treatment and Immunoprecipitation of [125I]TID-labeled PGHS-1—Following photolabeling of ovine vesicular gland microsomes with [125I]TID, the membranes were collected by centrifugation at 200,000 × g for 1 h and resuspended at a final protein concentration of 3 mg/ml. The resulting microsomal suspension was incubated with trypsin (10.3 microsomal protein:trypsin) for 15 min at 25°C. A 40-fold excess of trypsin inhibitor was then added, and the microsomal proteins were solubilized by addition of Tween 20 to a final concentration of 1%. The solution was sonicated for 30 s and centrifuged for 10 min at 10,000 × g. The solubilized protein was incubated with a mixture of antipeptide antibodies against the amino and carboxyl terminus of ovine PGHS-1 (20), and protein A-Sepharcl 4B was added. The immobilized protein A-antibody-PGHS-1 complex was collected by centrifugation at 1000 × g for 1 min and washed extensively with 0.1 M Tris-HCl, pH 7.4, containing 1% Tween 20 essentially as described previously (26). PGHS-1 was eluted from the complex by boiling the sample in SDS-PAGE-loading buffer. Samples were subjected to electrophoresis on 15% SDS-PAGE gels, and the proteins visualized by silver staining (27).

3rd-Labeled peptides were identified by autoradiography.

Proteolytic Digestion of [125I]TID-photolabeled PGHS-1 with Endoproteinase Lys-C—Following photolabeling, microsomes (600 μg of microsomal protein) were solubilized by the addition of Tween 20 to a final concentration of 1%, followed by sonication and centrifugation at 10,000 × g for 10 min. Solubilized PGHS-1 was immunoprecipitated using the mouse monoclonal antibody cyto-7 (26) coupled to Protein A-Sepharcl 4B. PGHS-1 was eluted from the Sepharcl beads by boiling in 0.5% SDS. The sample was diluted to 0.05% SDS in 10 mM Tris-HCl, pH 8.0,

RESULTS

Labeling of Ovine PGHS-1 by [125I]TID—PGHS-1 represents 5–10% of the protein present in ovine vesicular gland microsomes (26, 28). Ovine PGHS-1 can be cleaved by trypsin at one site, Arg277, generating a 33-kDa peptide which contains the amino terminus including the putative membrane binding domain between residues 70 and 117 and a 38-kDa peptide which contains the carboxyl terminus (29, 30). The intact 72-kDa PGHS-1 and both the 33-kDa amino-terminal and 38-kDa carboxyl-terminal tryptic peptides of PGHS-1 can be immunoprecipitated from solubilized microsomes using a combination of peptide-directed antibodies against the amino (residues 25–35) and carboxyl (residues 583–594) termini of ovine PGHS-1 (20) (Fig. 1); the intense bands between the 72- and 38-kDa bands seen upon silver staining of the immunoprecipitated PGHS-1 are due to IgG heavy chain.

Following photolabeling of ovine vesicular gland microsomes with [125I]TID, PGHS-1 was one of the most prominently radiolabeled proteins (Fig. 1). After tryptic digestion of photolabeled PGHS-1, both the 33-kDa amino-terminal and the 38-kDa carboxyl-terminal peptides were found to be labeled by [125I]TID. This result was unexpected because the putative membrane binding domain of PGHS-1 is located near the NH2-terminus, and it was anticipated that only the 33-kDa amino-terminal tryptic peptide would be labeled by [125I]TID. To determine if the labeling of either of the tryptic peptides was due to the presence of [125I]TID in the aqueous phase, photolabeling was performed in the presence of 20 mM reduced glutathione, which scavenges any [125I]TID present in the aqueous phase (22). Reduced glutathione did not affect the photolabeling of either tryptic peptide of PGHS-1 (Fig. 1), indicating that the photolabeling was not due to the presence of

![Fig. 1. Photolabeling of ovine PGHS-1 with [125I]TID.](image-url)
\[^{125}\text{I}]\text{TID in the aqueous phase. Competition for }^{125}\text{I}]\text{TID Photolabeling of PGHS-1 by Non-radioactive TID and NSAIDs—}^{125}\text{I}]\text{TID can nonspecifically photolabel regions of proteins present in the hydrophobic environment of the membrane; in addition, }^{125}\text{I}]\text{TID bound in a saturable manner to specific hydrophobic binding sites can photolabel regions of proteins which are not membrane-associated (24, 31, 32). To distinguish between these two possibilities, we examined the ability of nonradioactive TID to block photolabeling of PGHS-1 by }^{125}\text{I}]\text{TID. When }^{125}\text{I}]\text{TID labeling was performed in the presence of a 20-fold molar excess of nonradioactive TID, photolabeling of the 38-kDa carboxyl-terminal tryptic peptide of PGHS-1 was greatly diminished with little effect on the photolabeling of the 33-kDa amino-terminal tryptic peptide (Fig. 2). Silver staining established that the pre-treatment of microsomes with nonradioactive TID did not affect the generation of 38- and 33-kDa tryptic fragments from PGHS-1 (data not shown). The photolabeling of the 33-kDa tryptic peptide in the presence of nonradioactive TID is consistent with the association of a region or regions of this peptide with membranes. The competition of the }^{125}\text{I}]\text{TID photolabeling of the 38-kDa COOH-terminal tryptic peptide by nonradioactive TID suggests that the PGHS-1 protein contains a saturable, hydrophobic binding site(s) within the 38-kDa peptide which binds }^{125}\text{I}]\text{TID in the absence of competitor. Thus, photolabeling of the larger 38-kDa carboxyl-terminal peptide is not consistent with the association of this peptide with the ER membrane. The cyclooxygenase active site of PGHS-1 is a hydrophobic channel which contains segments of the peptide chains found in both the 33-kDa amino-terminal tryptic peptide and in the 38-kDa carboxyl-terminal tryptic peptide (19). We reasoned that the labeling of one or both of the tryptic peptides might result from the binding of }^{125}\text{I}]\text{TID within the cyclooxygenase active site. A comparison of the structures of }^{125}\text{I}]\text{TID and several NSAIDs suggest that a hydrophobic compound such as }^{125}\text{I}]\text{TID could occupy the cyclooxygenase active site (Fig. 3). Accordingly, the ability of two NSAIDs, ibuprofen and sulindac sulfide, to compete for labeling of PGHS-1 by }^{125}\text{I}]\text{TID was

![Fig. 2. Effect of nonradioactive TID on the photolabeling of ovine PGHS-1 by }^{125}\text{I}]\text{TID. Ovine vesicular gland microsomes were preincubated in the presence or absence of 100 }\mu\text{M nonradioactive TID prior to incubation and photolabeling with }^{125}\text{I}]\text{TID. After photolabeling, the microsomal samples were treated with trypsin and immunoprecipitated as described in the text. Immunoprecipitated PGHS-1 and PGHS-1 tryptic fragments were resolved on 15% SDS-PAGE gels and the gels exposed to film for autoradiography. Lane A, no pretreatment; lane B, 100 }\mu\text{M nonradioactive TID pretreatment.}

![Fig. 3. Structures of }^{125}\text{I}]\text{TID and several nonsteroidal anti-inflammatory drugs.}

\[^{125}\text{I}]\text{TID, ASPIRIN, IBUPROFEN, SULINDAC SULFIDE}
would compete with $^{125}$I TID for saturable binding sites, soning behind these experiments was that nonradioactive TID presence of excess non-radioactive TID (24, 31, 32). The rea-
L. Smith (unpublished observations).

examined (Fig. 4). Incubation of microsomes with ibuprofen or sulindac sulfide prior to photolabeling with $^{125}$I TID did affect the labeling pattern seen with ovine PGHS-1, decreasing somewhat the intensities of labeling of the 33-kDa tryptic peptide and essentially eliminating the labeling of the 38-kDa tryptic peptide. Pretreatment of PGHS-1 with the NSAIDs did not affect the generation of the 38- and 33-kDa peptides as determined by silver staining (data not shown). These results suggest that much of the photolabeling of PGHS-1 by $^{125}$I TID, particularly in the 38-kDa carboxyl-terminal tryptic peptide, is caused by the binding of $^{125}$I TID within the cyclooxygenase active site.

Photolabeling of Ovine PGHS-1 Occurs in a Region Containing the Proposed Membrane Binding Domain—Exhaustive digestion of $^{125}$I TID photolabeled ovine PGHS-1 with endoproteinase Lys-C generated a 20.5-kDa peptide which was reactive with an anti-peptide antibody raised against residues 25–35 of ovine PGHS-1 (Fig. 5); in contrast, this 20.5-kDa fragment was unreactive with an anti-peptide antibody raised against residues 203–217 of ovine PGHS-1 (data not shown). Importantly, this 20.5-kDa peptide was radiolabeled (Fig. 5). The molecular mass and immunoreactivity are completely consistent with this peptide being an endoproteinase Lys-C fragment, $^{3}$ Ala-Lys$^{166}$, derived from the NH$_2$ terminus of ovine PGHS-1.

**DISCUSSION**

$^{125}$I TID has been a useful tool for identifying transmembrane domains of proteins (22–24). $^{125}$I TID partitions efficiently into membrane lipid bilayers, and photolabeling of proteins with $^{125}$I TID occurs predominately in domains which are in direct contact with membranes (22). There is, however, a precedent for the incorporation of $^{125}$I TID into regions of a protein which are not in contact with the lipid bilayer. Photolabeling of the nicotinic acetylcholine receptor with $^{125}$I TID could be partially inhibited by receptor agonists and antagonists apparently because these agents exclude $^{125}$I TID from the receptor binding site (31). In order to distinguish between the photolabeling of membrane-associated regions of the nicotinic acetylcholine receptor and photolabeling of the receptor binding site, $^{125}$I TID photolabeling was performed in the presence of excess non-radioactive TID (24, 31, 32). The reasoning behind these experiments was that nonradioactive TID would compete with $^{125}$I TID for saturable binding sites, whereas nonradioactive TID could not compete for photolabeling of membrane-associated domains because the membranes could not be saturated with TID at submillimolar levels (24, 31, 32).

We have found that $^{125}$I TID is a useful probe for analyzing the structure of ovine PGHS-1. The photolabeling of ovine PGHS-1 is similar to that of the nicotinic acetylcholine receptor in that PGHS-1 was photolabeled by $^{125}$I TID in both a non-specific manner consistent with the association of regions of the enzyme with the ER membrane and in a specific manner consistent with the occupation of a hydrophobic pocket in the enzyme by $^{125}$I TID. Our data suggest that a region of ovine PGHS-1 contained in the NH$_2$-terminal 277 amino acids is associated with the ER membrane and that the COOH-termi-

**FIG. 5. Cleavage of $^{125}$I-photolabeled ovine PGHS-1 with endoproteinase Lys-C.** Ovine vesicular gland microsomes were incubated with $^{125}$I TID and photolabeled as described in the legend to Fig. 1. PGHS-1 was immunoprecipitated from solubilized microsomes, denatured, and subjected to exhaustive digestion with endoproteinase Lys-C as described in the text. Samples were resolved on 15% SDS-PAGE gels, and the gels were silver stained and exposed to film for autoradiography (right panel). Lane A, photolabeled microsomes; lane B, endoproteinase Lys-C digest of immunoprecipitated, photolabeled PGHS-1.

$^{3}$ This antibody was prepared and characterized by L. C. Hsi and W. L. Smith (unpublished observations).
structure of detergent-solubilized PGHS-1. Finally, [125I]TID may prove to be a valuable tool for comparing structural differences between the cyclooxygenase active sites of PGHS-1 and -2. Recent studies have indicated that isozyme specific NSAIDs can be developed (10, 11, 33, 34), and comparison of the labeling of the active sites may reveal some of the differences in the structures of the active sites of the isozymes which lead to NSAID selectivities (33–35).

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