The topology of association of the monotypic protein cyclooxygenase-2 (COX-2) with membranes has been examined using EPR spectroscopy of spin-labeled recombinant human COX-2. Twenty-four mutants, each containing a single free cysteine substituted for an amino acid in the COX-2 membrane binding domain were expressed using the baculovirus system and purified, then conjugated with a nitroxide spin label and reconstituted into liposomes. Determining the relative accessibility of the nitroxide-tagged amino acid side chains for the solubilized COX-2 mutants, or COX-2 reconstituted into liposomes to nonpolar (oxygen) and polar (NiEDDA or CrOx) spin relaxants allows us to map the topology of COX-2 interaction with the lipid bilayer. When spin-labeled COX-2 was reconstituted into liposomes, EPR power saturation curves showed that side chains for all but two of the 24 mutants tested had limited accessibility to both polar and nonpolar paramagnetic relaxation agents, indicating that COX-2 associates primarily with the interfacial membrane region near the glycero backbone and phospholipid head groups. Two amino acids, Phe66 and Leu67, were readily accessible to the non-polar relaxer oxygen, and thus likely inserted into the hydrophobic core of the lipid bilayer. However these residues are co-linear with amino acids in the interfacial region, so their extension into the hydrophobic core must be relatively shallow. EPR and structural data suggest that membrane interaction of COX-2 is also aided by partitioning of 4 aromatic amino acids, Phe59, Phe66, Tyr76, and Phe84 to the interfacial region, and by the electrostatic interactions of two basic amino acids, Arg62 and Lys64, with the phospholipid head groups.

Cyclooxygenases (COX)1-2 and -2 (also prostaglandin endoperoxide H2 synthase, PGHS-1 and -2) are the central enzymes in the pathway for the conversion of arachidonic acid to the biologically active prostaglandins and thromboxane (1). The COXs have been studied extensively because of their essential and regulatory role in prostaglandin synthesis and also because they are the site of action of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, Ibuprofen, Naproxin, Vioxx® and Celebrex®.

COX-1 and -2 are classified as integral membrane proteins because they can be solubilized only by treatment of membranes with detergent and not with chaotropic salts. These isozymes are localized to the luminal surfaces of the endoplasmic reticulum and the inner and outer membranes of the nuclear envelope (2). X-ray crystallographic studies have shown that that COX-1 and -2 are homodimers (3). Each monomer contains an epidermal growth factor domain at the N terminus followed by a membrane binding domain and a large globular catalytic domain (Fig. 6A). Membrane binding domains of both COX-1 and -2 contains four short, consecutive, amphipathic α-helices (A, B, C, and D) (Fig. 6A). Crystal structures of the cyclooxygenases indicate that basic, hydrophobic, and aromatic residues in the membrane binding domain are orientated away from the protein and form a surface on the enzyme that has been proposed to interact with the lipid bilayer. Constructs containing the COX signal sequences, as well as the epidermal growth factor and membrane binding domains linked to green fluorescent protein target these chimeras to the same subcellular membranes as native enzyme (4) demonstrating that the membrane binding domain is responsible for membrane association. Labeling studies with the lipid soluble 3-trifluoro-3-(m-[125I]iodophenyl)diazirin suggest that the membrane binding domain penetrates the hydrophobic core of the membrane (5), however the topology of membrane association of the cyclooxygenases has never been determined.

One method to investigate the topology of membrane proteins is site-directed spin labeling and power saturation EPR spectroscopy (6). This technique uses mutated recombinant proteins that contain a single reactive amino acid, usually cysteine, which can be conjugated with a paramagnetic spin label. Once reconstituted into liposomes these spin labels act as EPR probes that report on the environment of the modified amino acid side chain. The relative accessibility of spin label to a non-polar spin relaxation agent (oxygen) and to water soluble polar spin relaxants (NiEDDA or CrOx) can be used to determine whether the amino acids are within the lipid bilayer, or if they are solvent accessible. EPR spectroscopy to determine the paramagnetic spin relaxant accessibility of 24 single cysteine spin labeled COX-2 mutants reconstituted into liposomes, has allowed us to map the topology of COX-2 interaction with membranes.
**EXPERIMENTAL PROCEDURES**

*Materials*—1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-[phospho-1-serine] (Sodium Salt) (DOPS) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Oleic acid (99%) was purchased from Fluka. All restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA). Ni-NTA was purchased from Qiagen Inc. (Valencia, CA). Tween™-20 (10% solution) was obtained from Pierce. The nitroxide spin label (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)-methanethiosulfonate (MTSSL) was purchased from Reanal Fine Chemical (Budapest, Hungary). Arachidonic acid was obtained from Cayman Chemicals (Ann Arbor, MI). DPPH (α,α'-diphenyl-β-picrylhydrazyl) and CrOx (potassium tris(oxalato)chromate(III)) were purchased from Sigma.

NiEDDA was prepared as follows: equimolar amounts of Ni(OH)₂ (464 mg) and EDDA (881 mg) were mixed in 100 ml of 50% methanol in water. The mixture was stirred for 24 h at room temperature and an additional 24 h at 60 °C. The resulting solution was filtered, and the solvent evaporated to give 1.12 g of blue powder that by mass spectroscopic analysis showed a single peak (m/z 231) corresponding to the molecular ion of [NiEDDA-1H]⁺.

*Construction of His-tagged and Cysteine Mutants of PGHS-2*—The construction of baculoviral vectors for His-tagged human COX-2 and their expression in Sf-21 cells has been described previously (7). Two native cysteines in COX-2 that do not form disulfide bonds (positions 299 and 526)³ were mutated to serines in the histidine-tagged version of the human COX-2 protein using the QuikChange mutagenesis kit (Strategene). Using the His-tagged C299S/C526S COX-2 as a template, individual baculovirus for the triply substituted COX-2 proteins were created.

*Expression of Recombinant PGHS Protein in SF21 Insect Cells*—Spodoptera frugiperda (SF21) insect cells were grown in HyQ-SFX-Insect serum-free insect cell media (HyClone Logan, Utah) in 1-liter cultures at 27 °C in 2.8-liter Fernbach flasks (Bellco Biotechnology Inc., Vineland, NJ) shaken at 120 rpm. When cells reached a density of 1.5–2.0 x 10⁸ cells/ml, baculovirus were added at a multiplicity of infection of 1.0, and the infection was allowed to proceed for 72–96 h. Cells were harvested, washed with phosphate-buffered saline, and stored at −80 °C.

*Purification of His-tagged PGHS from Baculovirus-infected SF21 Insect Cells*—The COX-2 proteins were purified using nickel affinity chromatography from SF21 cells as described previously (7). Fractions with high specific cyclooxygenase activity were pooled and concentrated using an Amicon³ Ultra centrifugal filter device with a 30,000 molecular weight cutoff (Millipore) to 4 mg/ml. Cyclooxygenase protein was 80–90% pure following this step and had specific activities of ~25,000 units/mg for the C299S/C526S COX-2 enzyme, where 1 unit equals utilization of 1 nm O₂/min at 37 °C under standard polarimetric assay conditions (7). The various mutants had 10–100% of the activity of the C299S/C526S COX-2 enzyme (Table 1).

*Spin Labeling*—Nickel affinity-purified COX-2 (4 mg/ml) containing a single free cysteine was incubated with a 20-fold molar excess of MTSSL overnight at 4 °C. Unreacted MTSSL was removed by 4 consecutive dialyses against 4 liter of 25 mM Tris-Cl, pH 7.4 containing 50 mM KCl using a Slide-A-Lyzer® dialysis unit with a 10,000 molecular weight cutoff (Pierce). The spin-labeled protein was then concentrated to 15–20 mg/ml using an Amicon³ ultra centrifugal filter device with 30,000 molecular weight cutoff (Millipore). Previous spin counting studies using the nitroxide labeled H75C mutant has demonstrated 95–100% efficiency of labeling by this procedure without a noticeable decrease in the specific activity of the COX-2 (7).

*Preparation of Large Unilamellar Vesicles*—Large unilamellar vesicles (LUVs) were prepared from a mixture of DOPC: DOPS (7:3 mol/mol) and oleic acid (9.2%, w/w) (7). Aliquots of the phospholipids and oleic acid in chloroform were mixed and dried under a stream of nitrogen. The phospholipid film was redissolved in 0.5 ml of hexane, and the organic solvent was removed again under a stream of nitrogen. The resulting film was hydrated in a buffer of 25 mM Tris-Cl, pH 7.4 containing 50 mM KCl for 1 h at a concentration of 38 mM, then vortexed thoroughly and extruded 20 times through a 0.1-μm pore diameter polycarbonate filter using a Mini-Extruder (Avanti Polar Lipids). This process produces a transparent milky suspension.

*Incorporation of COX into the Preformed Liposomes*—Purified COX (20–30 nmol, 20 mg/ml) in 25 mM Tris-Cl, pH 7.4 containing 50 mM KCl was added to the preformed liposomes at a protein:phospholipid molar ratios of 1:500 in a total volume of 0.5 ml and incubated for 30 min at 37 °C (7). Proteoliposomes were next separated from free COX-2 by a density centrifugation. The liposome suspension was combined with 1 ml of 30% (w/v) Ficoll in 25 mM Tris-Cl and 50 mM KCl, pH 7.4 to give a final concentration of 20% (w/v) Ficoll and transferred to a 5-ml ultracentrifuge tube. 3 ml of 10% (w/v) Ficoll in 25 mM Tris-Cl, pH 7.4 containing 50 mM KCl was layered onto the 20% Ficoll liposome suspension, and the tube was filled with 25 mM Tris-Cl, pH 7.4 containing 50 mM KCl. Gradients were centrifuged at 45,000 rpm for 30 min at 20 °C in a Beckman SW 50.1 rotor. Liposomes and proteoliposomes band together at the interface between the 0% Ficoll and 10% Ficoll layers and were removed with a needle. Unincorporated protein remains in the lower 30% Ficoll layer. Typically 50% of the protein is incorporated into the liposomes under these conditions, and recovery of total activity in the gradient was 95–100% (7). Gold labeling of the proteoliposomes using anti-COX-2 antibody indicates that COX-2 is homogeneous and does not aggregate when reincorporated into the liposomes (7). For measurements with soluble paramagnetic reagents, the proteoliposome samples were

³The membrane binding domain is the most dissimilar region between the cyclooxygenase isozymes. There are deletions and additions between the COX-1 and COX-2 enzymes in the membrane binding domain that confound the concordance between the two sequences. Thus, the human COX-2 amino acid sequence numbering is used in this article rather than the conventional ovine COX-1 numbering system.
Membrane Topology of COX-2

added to dried CrOx or NiEDDA to achieve a final concentration of 10 or 50 mM, respectively.

**CW EPR Power Saturation Measurements**—Continuous wave (CW) EPR spectroscopy was performed on a Bruker ESP 300E spectrometer at room temperature (23 ± 2 °C) equipped with a two loop one gap resonator (Medical Advances, Milwaukee, WI). Samples were loaded into a gas permeable TPX capillary (Jagmar Ltd., Krakow, Poland) with total volume of 4 μL. Power saturation experiments were carried out using 2 Gauss modulation amplitude and varying microwave power (0.1–36 milliwatt). The scan range of all spectra was 100 Gauss and the microwave power required to reduce the resonance amplitude to half of its unsaturated value, and $\epsilon$ is a measure of the homogeneity of the saturation of the resonance. In this fit, $I$, $\epsilon$, and $P_{1/2}$ are adjustable parameters and yield a characteristic $P_{1/2}$. Values for $P_{1/2}$ were then generated for each sample under three different conditions: 1) equilibrated with nitrogen; 2) equilibrated with 20% oxygen (air); and 3) equilibrated with nitrogen in the presence of 10 mM CrOx or 50 mM NiEDDA. The samples were purged with gas for at least 20 min before performing each measurement. The resonator remained connected to the gas during the measurements. From these values of $P_{1/2}$, a collision parameter for O$_2$ (II(oxygen)) was determined according to Equation 2.

$$I(\text{oxygen}) = \frac{\Delta P_{1/2}(O_2)}{P_{1/2}(\text{DPPH})} = \frac{[P_{1/2}(O_2) - P_{1/2}(N_2)]/\Delta H_{pp}}{P_{1/2}(\text{DPPH})/\Delta H_{pp}(\text{DPPH})}$$

(\text{Eq. 2})

$\Delta H_{pp}$ is the peak-to-peak line width for the central resonance of the EPR spectra. A similar expression can be written for II(NiEDDA) or II(CrOx), the collision parameters for NiEDDA and CrOx. $P_{1/2}(\text{DPPH})$ and $\Delta H_{pp}(\text{DPPH})$ were obtained for a crystal sample of DPPH ($\alpha,\alpha'$-diphenyl-$\beta$-picrylhydrazyl).

The contrast function $\Phi$ is a variable useful for identifying residues that are lipid embedded or in loops (8). It is defined in Equation 3.

$$\Phi = \ln\left(\frac{I(O_2)}{I(\text{NiEDDA})}\right)$$

(\text{Eq. 3})

**RESULTS**

Site-directed spin labeling requires that a protein contain only a single reactive cysteine; therefore, a cysteine-less COX-2 template was prepared by mutating to serines the two cysteines (229 and 526) that do not form disulfide bonds (9). This cysteine-less template retained 70% of both cyclooxygenase and peroxidase activities compared with the native COX-2, so these mutations only minimally distort the structure of the enzyme. In contrast, serine substitution of the analogous cysteines in COX-1 significantly reduces cyclooxygenase activity (10). The cysteine-less COX-2 template also contains six histidines distal to the signal cleavage site, a modification that does not affect the activity of the enzyme and enables rapid purification (11).

The membrane binding domain was originally described as containing four helices (designated A–D) (3). However structural studies have since indicated that helix D is primarily a structural component of the cyclooxygenase active site (Fig. 6A). Thus, the twenty-four single cysteine mutants constructed for these studies were from the helices A, B, and C (Table 1). The histidine-tagged mutant proteins were expressed in sf-21 cells using baculovirus constructs and purified using nickel affinity chromatography (11). All of the single cysteine mutants retained significant cyclooxygenase activities (20–70% of the native) except for the W85C, which had 10% activity (Table 1). Conjugation of the cysteines with the spin label MTSSL did not further decrease cyclooxygenase activity. Previous studies with other proteins have demonstrated that conjugation with MTSSL rarely disrupts the native structure of cysteine-substituted proteins (12).

To examine the interaction of the COX-2 protein with membranes we examined three parameters: 1) the relative accessibility of nitroxide side chains of the MTSSL-conjugated, liposome-reconstituted COX-2 to nonpolar (O$_2$) and polar (NiEDDA) paramagnetic relaxation reagents using continuous wave power saturation EPR spectroscopy to determine the local

**TABLE 1**

| Relative cyclooxygenase activities of the triply substituted hCOX-2 cysteine mutants |
|---------------------------------|-----------------|-----------------|
| His-COX-2 mutant               | Relative activity before conjugation with MTSSL | Relative activity after conjugation with MTSSL |
| Native                          | 100             | NA              |
| C299S/C526S                    | 70              | NA              |
| F59C                           | 50              | 50              |
| L60C                           | 70              | 70              |
| T61C                           | 60              | 60              |
| R62C                           | 50              | 50              |
| I63C                           | 70              | 70              |
| K64C                           | 70              | 70              |
| L65C                           | 70              | 70              |
| F66C                           | 50              | 50              |
| L67C                           | 40              | 40              |
| N72C                           | 60              | 60              |
| T73C                           | 40              | 40              |
| V74C                           | 60              | 60              |
| H75C                           | 70              | 70              |
| Y76C                           | 70              | 70              |
| F77C                           | 60              | 60              |
| L78C                           | 30              | 30              |
| G83C                           | 35              | 35              |
| F84C                           | 50              | 50              |
| W85C                           | 10              | 10              |
| N86C                           | 50              | 50              |
| V87C                           | 30              | 30              |
| V88C                           | 30              | 30              |
| N89C                           | 30              | 30              |
| N90C                           | 20              | 20              |

* Cyclooxygenase activity determined by oxygen electrode as described previously on purified enzyme before and after conjugation with MTSSL. All activities are normalized to the specific activity of the His$_6$-tagged native COX-2.

* NA, not applicable.

* This His$_6$-tagged cysteine-less mutant has the two free cysteines present in the native enzyme, at positions Cys$^{299}$ and Cys$^{526}$, substituted with serines and is the parent enzyme for all the single cysteine substitutions listed below.
environment of helix A, B, and C amino acids; 2) the relative accessibility of the nitroxide side chains of MTSSL-conjugated solubilized, and liposome-reconstituted COX-2 to the charged polar paramagnetic relaxation agent CrOx, using continuous power saturation EPR spectroscopy, to determine how membrane association changes the accessibility of these amino acid side chains; and 3) the line width (\(\Delta H\)) and hyperfine splitting of EPR spectra of the solubilized MTSSL-conjugated COX-2 mutants in 30% sucrose to determine relative side chain mobility and helix main chain dynamics.

For all experiments, each of the 24 cysteine-substituted proteins was purified, conjugated with MTSSL, and concentrated (20 mg/ml, 280 \(\mu\)M). For proteoliposome experiments, spin-labeled COX-2 was then incorporated into preformed liposomes, and the proteoliposomes were separated from unincorporated COX-2 using a Ficoll density gradient centrifugation (7). Proteoliposomes were concentrated by ultracentrifugation and loaded into gas permeable TPX capillary tubes in a total volume of 4 \(\mu\)l. EPR spectroscopy was performed on a Bruker ESP 300E spectrometer at room temperature (23 \(\pm\) 2°C) equipped with a two loop one gap resonator. For power saturations experiments, peak to peak amplitude of the first derivative \(m_1 - \sigma\) resonance line (A) was measured and plotted against the square root of the incident microwave power (Fig. 1A). Values for \(P_{1/2}\) were then generated for the spin-labeled proteoliposome equilibrated with nitrogen, the spin-labeled proteoliposome equilibrated with 20% oxygen (air), or equilibrated with nitrogen in the presence of 50 mM NiEDDA or 10 mM CrOx (Fig. 1B).

**Continuous Wave Power Saturation EPR Spectroscopy of the Nitroxide-labeled COX-2 Reconstituted into Liposomes: Accessibilities to Oxygen and NiEDDA**—The solvent exposure of a nitroxide spin label attached to a cysteine residue in a protein can be measured by examining its collision frequency with another paramagnetic reagent such as oxygen or NiEDDA. The collision frequency of a nitroxide probe with paramagnetic relaxers depends on the product of the paramagnetic species translational diffusion coefficient and local concentration. Relative collision frequencies are determined by the method of continuous wave power saturation. The increase in \(P_{1/2}\) that occurs in the presence of NiEDDA, CrOx, or air is converted into a dimensionless accessibility parameter \(\Pi\), which is proportional to the collision frequency of the nitroxide with the respective paramagnetic reagent (13). The accessibility to soluble NiEDDA and CrOx is high for protein surface residues and low for amino acids in the protein interior and in the lipid bilayer. In contrast, oxygen is poorly soluble in aqueous solutions, but its solubility and diffusion coefficient are high in the bilayer. Oxygen is also more accessible in the protein interior than NiEDDA. When used in conjunction with structural information, \(\Pi\) values, and the derived contrast function \(\Phi\) are useful in identifying membrane imbedded residues and to differentiate between surface residues and those imbedded within the protein interior (8).

The accessibility parameter values for COX-2 reconstituted into liposomes and measured in the presence of \(N_2\) and NiEDDA (\(\Pi\)(NiEDDA)), and in equilibrium with \(O_2\) (\(\Pi\)(O\(_2\))) (Table 2) were plotted as a function of sequence position for helices A, B, and C (Fig. 2). The nine amino acids in Helix A show a periodic change in accessibility to both oxygen and NiEDDA with accessibility maxima at positions L60R1, T61R1, and K64R1, and accessibility minima at F59R1 and R62R1 (Fig. 2A). This periodicity is roughly 3.6, as would be predicted for an alpha helix. The phases of accessibility for oxygen and NiEDDA are synchronous for amino acids 59–65. The low \(\Pi\) values for both oxygen and NiEDDA indicate amino acids 59–65 do not enter the hydrophobic core of the lipid bilayer and are not solvent accessible either. One probable explanation for this limited accessibility to both paramagnetic reagents is that these 7 amino acids associate with the glycerol backbone and phospholipid head groups, and their close interaction at the membrane interface excludes both polar and nonpolar paramagnetic agents. Those amino acids with the lowest \(\Pi\) values for oxygen
Membrane Topology of COX-2

| Mutants | Ph (N2) | Ph (Air) | Ph (50 mM NiEDDA) | ΔHpp | II (Air) | II (NiEDDA) |
|---------|---------|----------|-------------------|------|----------|-------------|
| Helix A |         |          |                   |      |          |             |
| F9C     | 4.61    | 6.93     | 12.61             | 4.64 | 0.05     | 0.17        |
| L60C    | 4.72    | 11.32    | 14.11             | 3.48 | 0.19     | 0.27        |
| T61C    | 5.38    | 10.42    | 15.59             | 3.92 | 0.13     | 0.26        |
| R62C    | 4.29    | 8.07     | 11.61             | 4.50 | 0.08     | 0.19        |
| L63C    | 4.52    | 9.20     | 11.61             | 4.06 | 0.12     | 0.18        |
| K64C    | 4.61    | 9.48     | 12.25             | 3.77 | 0.13     | 0.20        |
| L65C    | 4.78    | 9.34     | 12.55             | 3.92 | 0.12     | 0.20        |
| F66C    | 4.09    | 8.82     | 7.15              | 3.48 | 0.14     | 0.09        |
| L67C    | 4.42    | 9.60     | 7.28              | 3.05 | 0.17     | 0.09        |
| Helix B |         |          |                   |      |          |             |
| N72C    | 3.73    | 7.45     | 6.67              | 4.29 | 0.09     | 0.07        |
| T73C    | 4.24    | 7.43     | 11.75             | 3.85 | 0.08     | 0.20        |
| V74C    | 5.77    | 7.95     | 13.51             | 4.62 | 0.05     | 0.17        |
| F75C    | 4.58    | 8.03     | 10.12             | 2.86 | 0.12     | 0.19        |
| Y76C    | 4.58    | 8.18     | 13.33             | 4.62 | 0.09     | 0.19        |
| I77C    | 5.38    | 8.54     | 12.62             | 4.07 | 0.07     | 0.18        |
| L78C    | 6.02    | 8.85     | 13.14             | 3.63 | 0.08     | 0.20        |
| Helix C |         |          |                   |      |          |             |
| G63C    | 3.99    | 6.00     | 10.76             | 4.62 | 0.04     | 0.15        |
| F64C    | 3.66    | 5.88     | 9.29              | 4.95 | 0.04     | 0.11        |
| W65C    | 4.37    | 6.88     | 11.37             | 5.17 | 0.04     | 0.14        |
| N66C    | 3.76    | 6.90     | 11.71             | 4.18 | 0.08     | 0.19        |
| V67C    | 3.43    | 8.55     | 12.13             | 3.08 | 0.17     | 0.28        |
| V68C    | 5.64    | 8.17     | 10.02             | 4.4  | 0.06     | 0.10        |
| N69C    | 3.11    | 6.26     | 5.58              | 4.51 | 0.05     | 0.06        |
| N90C    | 4.85    | 7.57     | 11.28             | 4.07 | 0.07     | 0.16        |

The II values determined using the CrOx and NiEDDA paramagnetic reagents are not directly comparable because different concentrations were used; 10 ms for CrOx and 50 ms NiEDDA. Also, NiEDDA is neutral and CrOx has a net negative charge, so the II values for CrOx are higher for basic amino acids. CrOx was used instead of NiEDDA to replicate the EPR experiments of Lin et al. (23) to estimate distances of individual amino acids in sPLA2 from membranes and thus orientate this enzyme with respect to the bilayer. However, hydrophobic surface-exposed residues on the solubilized COX-2 exhibited relatively low accessibility to CrOx, presumably because of detergent masking, making distance estimates impossible for COX-2. (The sPLA2 enzyme studied by Lin et al. (23) is soluble, and EPR analysis is possible in the absence of detergents.) Gross differences in CrOx accessibility exist between the detergent-solubilized and liposome-reconstituted COX-2 enzymes, demonstrating that interaction the liposome restricts accessibility to water soluble paramagnetic reagents.

and NiEDDA, F59R1, and R62R1, would be predicted to be the most closely associated within the interfacial region. The greater accessibility to oxygen compared with NiEDDA (Φ > 0) of two amino acids in helix A, F66R1 and L67R1, indicate these residues likely are inserted into the hydrophobic core of the lipid bilayer (Fig. 2A) (8). However, this insertion must be relatively shallow as these amino acids are co-linear with preceding amino acids 59–65 situated at the membrane interface.

To confirm that the limited accessibility of helix A side chains to soluble paramagnetic reagents is caused by membrane association and not the orientation of these residues within COX-2, we examined their accessibility to another aqueous paramagnetic agent, CrOx, comparing COX-2 in detergent micelles and COX-2 reconstituted into liposomes (Fig. 3). Accessibility to CrOx is significantly reduced in the liposome incorporated COX-2 compared with the solubilized COX-2 for all amino acids except L67R1, one of the two residues in Helix A whose accessibility to oxygen indicates it is embedded within the bilayer. The overall reduced accessibility to CrOx observed in liposomes compared with solubilized COX-2 demonstrates that binding of helix A to membranes limits paramagnetic reagent accessibility, and supports the hypothesis that residues with low accessibility to both oxygen and NiEDDA associate at the interface of the lipid bilayer (Fig. 3). The relative inaccessibility of Leu^67 to CrOx in both the detergent-solubilized and liposome-reconstituted COX-2 suggest this residue is in a region that binds both lipid and detergent.

The fractional solvent accessibilities (f_s) calculated from the crystal coordinates of the murine COX-2 (PDB entry 1CVU) (Fig. 2B) (8) and the orientation of helix A amino acids Phe^59, Arg^62, Ile^63, Phe^66, and Leu^67 in the crystal structure (Fig. 6B) indicate these residues are on the surface of COX-2 where they could interact with the lipid bilayer. The spectra of F59R1, R62R1, I63R1, F66R1, and L67R1 in 30% sucrose also show narrow central peaks (Fig. 5A) and modest anisotropic hyperfine splitting, indicating these amino acids have relatively high mobility in the solubilized COX-2, consistent with a position on the surface of the COX-2. That these surface exposed residues nevertheless have low accessibility to NiEDDA and CrOx when COX-2 is reconstituted into proteoliposomes is strong evidence that they participate in membrane binding. Thus, the structural and EPR accessibility data are both consistent with Phe^59, Arg^62, Ile^63 forming a contact surface at the interface of the lipid bilayer, and with Phe^66 and Leu^67 inserting shallowly into the hydrophobic core of the lipid bilayer.

Accessibility data for helix B show a different pattern than helix A. No obvious periodicity in II values is observed within this short helix. The paramagnetic reagent NiEDDA is more accessible than oxygen for all residues except the N72R1, which suggest that none of these amino acids are localized within the hydrophobic core of the lipid bilayer. The II(Oxygen) values for N72R1 are similar to other amino acids in this helix, but the II(NiEDDA) is much lower, increasing the Φ value to greater than zero (Fig. 2A). It seems likely that N72R1, which is located...
at the end of the loop that leads to helix B, is less accessible to NiEDDA than oxygen because it is buried within the protein, not because it is inserted within the bilayer (8).

The crystal structure of COX-2 shows that helix B is raised above the surface of a plane that helices A and C would rest (Fig. 6A) (14), suggesting it may be more tightly packed against the globular COX-2 catalytic domain than helices A and C. Close packing is supported from the EPR spectral data of nitroxide-labeled mutants of this helix (Fig. 5B). Most residues of helix B show broadening of the central peak and increased anisotropic hyperfine splitting, compared with residues in helix A, indicating more restricted motion of the nitroxide spin labels in this helix. Nevertheless, the CrOx accessibility data show reduced accessibility to paramagnetic reagents for helix B (Fig. 2A) when COX-2 is reconstituted into liposomes, indicating that that helix B must also bind close to the membrane interface. Amino acids Tyr76 and Ile77 of helix B are predicted to be on the surface of COX-2 (Fig. 2B) and could play a role in the membrane association at the interfacial region, but neither of these residues have Φ values typical of side chains inserted into the hydrophobic core of the lipid bilayer (Φ > 0) (8).

Paramagnetic accessibility for helix C shows the same 3.6-amino acid periodicity as helix A (Fig. 2A). Again the NiEDDA and oxygen accessibility are synchronized, but for this helix there are no residues whose oxygen accessibility exceeds that of NiEDDA. Thus, the data suggest that no helix C amino acids penetrate into the hydrophobic core of the bilayer. As with helices A and B, all the accessibility values (II) of these residues to the soluble CrOx paramagnetic reagent are reduced in the liposome-reconstituted COX2 compared with the solubilized enzyme (Fig. 3), indicating that association with membranes brings helix C into close enough contact with the membrane to exclude both polar and nonpolar paramagnetic relaxation agents. The EPR spectra of all amino acids substitutions, except V87R1 (Fig. 5C), show at least some central peak broadening and anisotropic hyperfine splitting; indicating that packing of this helix is similar to helix B. Phe84 (Fig. 2B) is located at a minimum for both II (oxygen) and II(NiEDDA), is on the surface helix C (Figs. 2 and 6C) and thus likely to aid COX-2 membrane association. Val87 is also on the surface of helix C (Figs. 2B and 6C), but the high accessibility of this residue to both NiEDDA and oxygen suggests it is not shielded by the membrane surface.

Qualitatively, the spectra for spin labeled mutants of helix A in 30% sucrose have uniformly sharp central features and a rel-
ative lack of anisotropic hyperfine splitting compared with those of helices B and C, indicating increased side chain mobility (Fig. 5). The differences in the sharpness of the central peaks of helix A compared with helices B and C is also evident in the plot of $\Delta H_0^{-1}$ calculated from the spectra of the nitroxide-labeled COX-2 mutants (Fig. 4). Because nitroxide rotation is limited because of hydrogen bonding between the disulfide linkage of MTSSL and the peptide backbone, increased mobility as measured by the inverse of the central peak width ($\Delta H_0^{-1}$) has been interpreted to correlate with local backbone flexibility (15). The increased local backbone flexibility in helix A compared with helices B and C suggests this helix does not pack as tightly against the COX-2 catalytic domain which may allow helix A to better conform to, or maintain contact with the fluid lipid bilayer. Flexibility may also allow this helix to tilt slightly and could explain how residues Phe$^{66}$ and Leu$^{67}$ insert into the hydrocarbon core of the membrane while Leu$^{60}$, Arg$^{62}$, and Ile$^{63}$ remain at the interfacial region.

With the exception of His$^{75}$ in helix B, and Val$^{87}$ in helix C, the spectra for spin-labeled mutants of helices B and C are generally broader and/or show increased anisotropic hyperfine splitting compared with spectra from helix A (Figs. 4 and 5). Such spectra are consistent with moderate to low side chain mobility indicative of increased side chain interactions or tighter packing of helices B and C against the COX-2 catalytic domain. Crystal structures (Fig. 6A) show that helix B is situated above a plane formed by the axis of helices A and C, indicating it packs more tightly against the catalytic domain. In agreement with this structural data, no clear periodicity for paramagnetic accessibility for helix B is observed, suggesting this helix doesn’t interact as closely with the membrane surface as helices A and C.

**DISCUSSION**

Our data obtained using EPR power saturation analysis to determine the local environments of amino acids in the COX-2 membrane binding domain indicate this cyclooxygenase isozyme localizes primarily to the membrane interfacial region. Our studies agree with earlier experiments which used fluorescent spectroscopy to determine the local environment of tryptophan side chains for Class A $\alpha$-helical amphipathic peptides and that concluded these peptides insert only to the depth of the phospholipid ester linkages (16). In contrast, x-ray diffraction studies with other model class A amphipathic peptides indicated these peptides penetrated into the hydrocarbon core of the lipid bilayer (17). It seems likely that the depth of membrane insertion of amphipathic $\alpha$-helices may vary depending on the composition and orientation of aromatic, hydrophobic and basic amino acids along the helix, which would allow their corresponding proteins to have different functions.

These experiments show that only two amino acids of COX-2, Phe$^{66}$ and Leu$^{67}$, have accessibility parameters typical of residues that are inserted within the hydrophobic core of the lipid bilayer. Because these two residues are collinear with the preceding amino acids in helix A that localize to the interfacial region, the membrane penetration of Phe$^{66}$ and Leu$^{67}$, and the entire COX-2 into the hydrophobic core must be relatively shallow.

It seems likely that the aromatic amino acids are important contributors to COX-2 membrane association. Phe$^{59}$, Tyr$^{76}$, and Phe$^{84}$ are on the surface of COX-2 (Figs. 2B and 6B) and have limited accessibility to both polar and nonpolar paramagnetic relaxation agents when the enzyme is reconstituted into liposomes (Figs. 2 and 3). Aromatic amino acids preferentially partition to the interfacial rather than the hydrocarbon core of bilayers, and are commonly found at the solvent-bilayer interface of transmembrane helices of integral membrane proteins (18). Thus, the physical properties of aromatic amino acids, the orientation of these specific residues on the COX-2 surface, and our EPR power saturation data are all consistent with Phe$^{59}$, Tyr$^{76}$, and Phe$^{84}$ facilitating COX-2 membrane binding at the interfacial region.

Two basic amino acids, Arg$^{62}$ and Lys$^{64}$, and are also exposed on the surface of COX-2 (Figs. 2 and 6B) and similarly shielded...
from polar and nonpolar paramagnetic reagents. Ionic interactions between these side chains and the phospholipid head groups also likely stabilize binding of COX-2 to membranes.

Although one might hypothesize that the hydrophobic amino acids Leu^60, Leu^65, Val^74, Ile^77, Ile^78, Val^87, and Val^96 in the helices A–C play a role in membrane interaction, the accessibility data (II values) indicate they do not penetrate into the hydrophobic core of the lipid bilayer, nor are they necessarily positioned to interact with phospholipid head groups. Hydrophobic amino acids such as leucine, valine, and isoleucine are more often observed in the hydrophobic core of transmembrane helices or in the interior of proteins. Thus, rather than playing important roles in membrane association, these hydrophobic residues more likely facilitate packing of the membrane binding domain helices against the COX-2 catalytic domain, or provide a hydrophobic environment for substrate entry within the mouth of the cyclooxygenase active site.

Helix A appears to have the most prominent role in membrane binding. Earlier mutational studies have demonstrated the relative importance of helix A for cyclooxygenase activity (19). Mutations that substitute two tryptophans, an isoleucine and leucine in helix A completely inactivated COX-1, while mutants with similar substitutions in the other two helices retained significant (helix B) or partial (helix C) activity. Helix A contains three amino acids that contact the phospholipid head groups (Phe^69, Arg^62, and Lys^64) and two (Phe^66 and Leu^67) that insert into the hydrophobic core of the bilayer.

It has been previously observed that the amino acids sequences of the membrane binding domains of COX-1 and COX-2 are poorly conserved (Fig. 7). Importantly, many of the residues we have identified in these studies as important for membrane association of COX-2 are not conserved in COX-1. Phe^59, Arg^62, and Phe^66 in helix A of COX-2 are nonconservatively substituted with Ile/Leu, Trp, and Ser/Thr, respectively, in helix A of COX-1. Nevertheless, there is some sequence conservation between the two isozymes. Ile^63 and Lys^64 in COX-2 are leucine and arginine in COX-1, and the membrane inserted Leu^67 is invariant in all cyclooxygenases. However, the phenylalanine preceding Leu^67 in COX-2 is a serine or threonine in COX-1, which might prevent the COX-1 amino acid pair from inserting into the hydrophobic core of the lipid bilayer. The weak conservation of amino acids important for membrane association between the cyclooxygenase isozymes may signify that relatively nonspecific combinations of aromatic and basic amino acids properly orientated are all that is necessary for cyclooxygenase membrane association. That the overall aromaticity of helix A is conserved between the COX-1 and -2 supports this view. Phe^59 and Phe^66 in COX-2 are compensated for by Trp^60 and Trp^62 in COX-1 (Fig. 7). A more provocative possibility is that the dissimilarity of membrane binding domain sequence between COX-1 and -2 may result in: 1) different topologies of membrane association for the two cyclooxygenases; 2) localization of the two isozymes to membranes with different lipid compositions; or 3) differential utilization of fatty acid substrates by the two cyclooxygenases. Any of these possibilities would have important implications for the biological activities of COX-1 and -2. Unfortunately, the sensitivity of COX-1 to cysteine substitution precludes similar EPR studies on this isozyme.

Another interesting observation from these experiments is the apparent inaccessibility of both nonpolar and water soluble paramagnetic reagents to amino acids of the membrane binding domain in the liposome-reconstituted COX-2. While steric factors likely limit access, it seems probable that the concentrations of oxygen, and NiEDDA and CrOx at the membrane interface are lower than in membranes or in bulk solution, and that the chemical environment of the “interfacial region” may also contribute to the observed “inaccessibility” (18). Thus, these experiments indirectly illustrate the unique nature of the interfacial region which is neither strictly polar or nonpolar, hydrophobic, or hydrophilic (18). The membrane binding domains of COX-2 forms the mouth of the cyclooxygenase active site which abuts the interfacial region. This orientation may facilitate diffusion of fatty acid substrates to the cyclooxygenase active site following release from membrane phospho-
FIGURE 5. A, CW-EPR spectra of nitroxide-labeled solubilized COX-2 helix A mutants in 30% sucrose. B, CW-EPR spectra of nitroxide-labeled solubilized COX-2 helix B mutants in 30% sucrose. C, CW-EPR spectra of nitroxide-labeled solubilized COX-2 helix C mutants in 30% sucrose. All spectra were collected as described under “Experimental Procedures.”
lipids if amphipathic polyunsaturated fatty acids also preferentially partition to the interfacial membrane region.

Labeling studies with the hydrophobic labeling reagent 125I-TID have provided physical evidence that both COX-2 and the cPLA2 C2 domain penetrate the lipid bilayer (19, 20). EPR experiments with the cPLA2 C2 domain, analogous to those reported here for COX-2, suggest that membrane association of this protein domain also depends on the insertion of two hydrophobic amino acids into the hydrocarbon core (21). One amino acid (N64R) was also inaccessible to oxygen and CROX and thus predicted to reside at the membrane interface in the membrane-associated enzyme. cPLA2 however is a soluble enzyme that only transiently interacts with membranes in response to calcium, while the cyclooxygenases are resident peripheral membrane proteins that require detergents for solubilization. The more extensive network of interacting aromatic and basic amino acids in the membrane binding domain of COX-2 provide a mechanism for the stable membrane binding of COX-2.

Our EPR studies show that the interfacial amino acids responsible for COX-2 membrane association are located at local minima for oxygen and NiEDDA accessibility (Fig. 2), and membrane inserted amino acids are located at local accessibility maxima for NiEDDA and local accessibility minima for oxygen. These results agree in part with Hubbel et al. (22) predictions, but this group envisioned that a surface-absorbed amphipathic helix would penetrate the hydrophobic core of the lipid bilayer along its entire length, and that the pattern observed would be that of NiEDDA and oxygen accessibility parameters that were 180° out of phase.

The interfacial localization of COX-2 likely has some biological significance for catalysis of acid polyunsaturated fatty acid substrates and for inhibition by acidic aromatic NSAIDs, as these compounds would be predicted to preferentially partition to the interfacial region of membranes where they would have ready access to the cyclooxygenase active site. The lack of conservation between COX-1 and -2 for amino acids important for membrane interaction of COX-2 may signify that these two isozymes associate with the membranes differently, or with different membranes systems, which may affect their catalytic properties and biological activities.

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Membrane Topology of COX-2

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