Comparison of Structural Stabilities of Prostaglandin H Synthase-1 and -2*

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There are two known isoforms of prostaglandin H synthase (PGHS), a key enzyme in the conversion of arachidonic acid to bioactive prostanooids. The “constitutive” isoform, PGHS-1, is thought to have housekeeping functions, and the “inducible” isoform, PGHS-2, has been implicated in cellular responses to cytokines. The two isoforms have high sequence conservation in the cyclooxygenase active site and quite similar crystallographic structures, but differ markedly in their interactions with many cyclooxygenase substrates and inhibitors. We have evaluated the stability of the overall folding, and of the active sites of ovine PGHS-1 and human PGHS-2 using denaturation with guanidinium hydrochloride (GdmHCl). Changes in hydrodynamic and cross-linking properties indicated a dimer → monomer transition for both isoforms between 0.5 and 2 M GdmHCl; the monomers unfolded at higher GdmHCl levels. Changes in overall secondary and tertiary structure, measured by tryptophan fluorescence and circular dichroism, occurred in two phases for each isoform, with the transition between the phases at 0.2–0.5 M GdmHCl. Disruption of active site functions (cyclooxygenase, peroxidase, and cyclooxygenase inhibitor binding activities) began at GdmHCl levels below 0.2 M. The structural and functional changes were completely reversible up to about 2 M GdmHCl, they were more pronounced at lower protein levels, and they required lower GdmHCl levels for PGHS-2 than for PGHS-1. The results are consistent with a four-state denaturation process for both isoforms: native dimers → inactive dimers → compact monomers → unfolded monomers. The first two steps are reversible for both isoforms; PGHS-2 undergoes the first and last steps more readily than PGHS-1. Thus, the structural stability of PGHS-2, both in the active site regions and in the subunits overall, is distinctly less than that of PGHS-1. These differences in structural stability may contribute to the isoforms’ active site ligand selectivity.

Prostaglandin H synthase (PGHS) catalyzes two key steps in prostaglandin biosynthesis: oxygenation and cyclization of arachidonic acid to form PGG2, and the reduction of the hydroperoxide of PGG2 to form PGH2. Two isoforms of PGHS have been described. PGHS-1 is believed to be a housekeeping enzyme, whereas PGHS-2 is thought to play important roles in cell proliferation and inflammation (1). Both isoforms have cyclooxygenase and peroxidase activity, with comparable specific activities for the purified recombinant enzymes (2). PGHS-1 and PGHS-2 have about 60% amino acid identity, with much higher conservation of active site residues (3). Crystallographic data for oPGHS-1 and hPGHS-2 revealed similar dimeric structures for the two isoforms, with a root-mean-square deviation of only 0.9 Å for backbone atoms (4–6).

Despite the similarities in the crystallographic structures, the two PGHS isoforms have been found to have different selectivities for fatty acid substrates and cyclooxygenase inhibitors (1). These specificities have been ascribed in part to a larger cyclooxygenase pocket in PGHS-2 (7). The observations that PGHS-2, but not PGHS-1, retains oxygenase activity and native tyrosyl radical after acetylation by aspirin (8–10) also indicate that PGHS-2 has a more accommodating cyclooxygenase active site. The presence of a smaller valine residue at position 509 in PGHS-2, instead of the isoleucine at the corresponding position (residue 523) in PGHS-1, does provide a significantly larger accessible volume in the upper part of the cyclooxygenase channel (5, 6). However, results from mutagenesis studies in both PGHS-1 and PGHS-2 indicate that the difference between valine and isoleucine at position 509/523 does not account for all the differences in inhibitor specificity between the isoforms (11–13).

As a first step in examining more dynamic aspects of the structural properties of the two isoforms, we have evaluated their susceptibilities to unfolding by denaturant. Controlled denaturation has proven a powerful approach to characterizing the folding stabilities of proteins in solution (14) and the contributions of oligomeric arrangements to overall structure (15). Results from present chemical denaturation studies indicate that the PGHS-2 structure, both overall and in the peroxidase and cyclooxygenase active sites, is less stable than in PGHS-1.

EXPERIMENTAL PROCEDURES

Materials—Glycerol, guaiacol, hydrogen peroxide, hemin chloride, D-tryptophan, NAc-Trp-OEt, lysozyme, standards for size exclusion chromatography, 25% glyceraldehyde solution, sodium deoxycholate, and NaBH4 were from Sigma. PD-10 desalting columns were from Amersham. D-tryptophan, NaBH4 to decompose hydroperoxides (16). Tween 20 and GdmHCl (Sequanl grade) were from Pierce. Sterile Acrodisc filters (0.45 μm) were from Gelman Sciences (Ann Arbor, MI).

PGHS-2 and PGHS-1 Expression and Purification—Recombinant hPGHS-2 was expressed in cultured insect cells using a baculovirus vector and purified to homogeneity as the apoenzyme essentially as described previously (17). Recombinant human PGHS-1 was expressed...
concentrations were determined by the modification of the Lowry assay from the membrane fraction by extraction with 1% Tween 20. oPGHS-1 isoforms and lysozyme was evaluated by solvent perturbation differences used the standard algorithm included in the polarimeter software. Exposure of tryptophan residues to solvent in the two PGHS isoforms, indicating a small increase in masking of the tryptophans by detergent. Lysozyme was examined as a control, and 

activity significantly.

Spectral Measurements—Electronic absorbance spectra were recorded in a 1-cm pathlength quartz cuvettes with a Shimadzu model UV-2101PC spectrophotometer thermostatted at 25 ± 0.1 °C. For critical measurements, the spectrophotometer was warmed up for 30 min before use and the base-line spectrum was scanned repeatedly to verify stable performance.

Fluorescence measurements were carried out with a SLM Aminco model SPF-500 fluorophotometer at room temperature. Inner filter effects were minimized by diluting protein solutions to reach an A290 of less than 0.05.

CD spectra of the PGHS apoenzymes (1.0 μM subunit) were recorded at a scan speed of 50 nm/min with a response time of 2 s and a 2.0 nm slit setting, in a 1-mm pathlength quartz cuvette at room temperature using a Jasco J700 spectropolarimeter. Estimations of secondary structures used the standard algorithm included in the polarimeter software.

Quantitation of Solvent-exposed Tryptophan Residues in PGHS-1 and—Exposure of tryptophan residues to solvent in the two PGHS isoforms and lysozyme was evaluated by solvent perturbation difference spectroscopy, using 20% glycerol as the perturbant (20). All solutions were prepared in high purity Milli Q water and passed through a 0.45 μm filter. The spectrophotometer cuvettes were carefully washed with a cotton swab moistened with dilute Tween 20 and then rinsed with high purity water. UV absorbance spectra were recorded for each protein (16.7 μM for oPGHS-1 and hPGHS-2 or 20 μM for lysozyme) in 0.1 M potassium phosphate, pH 7.2, containing the desired level of Tween 20 and either 0 or 20% v/v glycerol. The slit width was 2 nm, and the cuvettes were thermostatted at 25 ± 0.1 °C.

For each Tween level, the control spectrum was subtracted from the spectrum obtained with glycerol present to obtain a difference spectrum. The maximum in the NAc-Trp absorption spectrum was found at 292 nm. The fractional exposure of tryptophans by detergent. Lysozyme was examined as a control, and was found to have about three tryptophans exposed to solvent, with less exposure as the Tween concentration was raised (Fig. 1). Higher detergent levels decreased the tryptophan exposure somewhat for both PGHS.

RESULTS

Solvation Exposure of Tryptophan Residues in oPGHS-1 and hPGHS-2—oPGHS-1 has nine, and hPGHS-2 six, tryptophan residues (3, 30), which are potentially useful in fluorimetric monitoring of unfolding in these proteins. Solvent perturbation difference spectroscopy was used to determine how many of these tryptophan residues are exposed to solvent, and thus not likely to contribute significantly to the decrease in intrinsic fluorescence during denaturation. It was anticipated that the detergent used to solubilize the enzymes might give a variable level of masking of some tryptophan residues, particularly those in the putative membrane anchor region (4), so the tryptophan exposure was checked at several detergent levels. Approximately 1.2 tryptophan residues are exposed in oPGHS-1 and 2.9 tryptophan residues in hPGHS-2 were found to be exposed to solvent at 0.05% Tween 20 (Fig. 1). Higher detergent levels decreased the tryptophan exposure somewhat for both PGHS isoforms, indicating a small increase in masking of the tryptophans by detergent. Lysozyme was examined as a control, and was found to have about three tryptophans exposed to solvent, with less exposure as the Tween concentration was raised (Fig. 1), in accord with the report that four of the six tryptophans in

in insect cells2 using the methods described for PGHS-2, and solubilized from the membrane fraction by extraction with 1% Tween 20. oPGHS-1 was purified to homogeneity from sheep seminal vesicles (16). Protein concentrations were determined by the modification of the Lowry assay described by Peterson (18). The cytochrome oxidase specific activity ranged from 58 to 147 μg of O2/min/mg for oPGHS-1 and from 30 to 56 μg of O2/min/mg for hPGHS-2. The apoenzymes of both PGHS isoforms were used in these studies; heme was added only for assay of activity.

Activity Assays—Cytochrome oxidase activity was determined with an oxygen electrode (16) at 30 °C. The standard reaction mixture contained 0.1 mM potassium phosphate, pH 7.2, 5 mM d-trehalose, 0.1 mM arachidonic acid, and 1 μM heme. Peroxidase activity was measured spectrophotometrically at 436 nm in a 2 ml-reaction mixture containing 0.1 M Tris-HCl, pH 8.0, 0.5 mM guaiacol, 0.4 mM hydrogen peroxide, and 1 μM heme (19). Samples treated with GdmHCl were diluted at least 200-fold into the assay mixtures for determination of cytochrome oxidase or peroxidase activity; the residual denaturant was found not to affect the activity significantly.

Fraction exposed = \( \frac{A_{292} - A_{292}/A_{550}}{A_{292}/A_{550}} \times 100 \) (Eq. 1)

where \( A_{292} \) and \( A_{329} \) are the difference and direct absorbance values at 292 nm, respectively, for the protein or the model compound. The number of exposed tryptophans was obtained by multiplying the fractional exposure calculated from Equation 1 by the total number of tryptophans in each protein.

\( \text{GdmHCl Denaturation of PGHS Isoforms—} \)

Stock solutions of 6 or 8 M GdmHCl were prepared in 0.1 M potassium phosphate, pH 7.2. The pH of the stock solutions was adjusted as necessary with KOH. GdmHCl concentrations were determined from refractive index measurements of the stock solutions and from the refractive index increment of a series of standards (tryoglobin, apoferritin, catalase, transferrin, cytochrome c, and glycyl-tyrosine) using published values for the Stokes radii (24).

Cross-linking Studies—The procedures were adapted from those of White et al. (25). oPGHS-1 was diluted to 0.15 or 1.5 μM in 0.1 M potassium phosphate, pH 7.2, containing 0.1% Tween 20 and 0–3 M GdmHCl (total volume, 250 μl) and preincubated at room temperature for 30–45 min before addition of 20 μl of 25% glutaraldehyde. The reaction was quenched after 15 min by addition of 12 μl of 2 M NaBH4, and incubation for 20 min. The mixture was diluted by addition of water (0.75 ml), and the protein was precipitated by addition of 100 μl of 3% sodium deoxycholate and 100 μl of 72% trichloroacetic acid. The precipitate was collected by centrifugation at 13,000 rpm in a microcentrifuge at 0°C. The sample was washed once more with 1.75 ml of 72% trichloroacetic acid and resuspended at 13,000 rpm for 10 min. The precipitated proteins were dissolved in 20–40 μl of electrophoresis sample buffer and separated by electrophoresis under denaturing conditions in an 8% polyacrylamide gel (26). Protein bands were stained with Coomassie Blue R250 and quantitated by densitometry.

Cyclooxygenase Inhibitor Binding—Flurbiprofen and indomethacin are competitive, tight-binding, stoichiometric cyclooxygenase inhibitors for purified PGHS-1 and -2 (2, 4, 27, 28). The two PGHS isoforms exhibited emission maxima near 337 nm when excited at 282 nm. Equilibration of oPGHS-1 or hPGHS-2 with excess (3 eq) flurbiprofen led to a blue shift of 7–8 nm in the emission maximum and a 20% decrease in fluorescence intensity. Equilibration with excess (3 eq) indomethacin produced only small red shifts, but did decrease the fluorescence intensity by 47% in oPGHS-1 and by 42% in hPGHS-2. A similar effect of indomethacin on the hPGHS-2 fluorescence was reported recently by Houtzager et al. (29) and attributed to quenching by inhibitor bound in the vicinity of the tryptophans. To assess complex formation by flurbiprofen and indomethacin, PGHS samples were preincubated with the desired level of GdmHCl at room temperature for 30 min before addition of 3 eq of flurbiprofen or indomethacin. The incubation was then continued for an additional 40 min before measurements of the fluorescence intensity at 338 nm were made. This length of time was found to maximize cyclooxygenase inhibition, reflecting maximal complex formation. The intrinsic fluorescence intensities of unbound flurbiprofen and indomethacin were found to be constant over the range of 0–2 M GdmHCl.

2 Q. Guo and R. J. Kulmacz, unpublished results.

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lysozyme are exposed (20). Because few of the tryptophan residues in either isof orm are exposed to solvent, the proteins’ intrinsic fluorescence is likely to include contributions from many buried tryptophans distributed throughout the proteins. These results are consistent with oPGHS-1 crystallographic data (4), which show four of the nine tryptophan residues (at positions 75, 77, 98, and 100) on the surface. All four are in the proposed membrane anchor domain and are thus probably partially shielded by bound detergent. Only one of the oPGHS-1 surface tryptophans is conserved in hPGHS-2: residue 85, corresponding to residue 100 in oPGHS-1 (3). On the other hand, the five buried oPGHS-1 tryptophans are all conserved in hPGHS-2 (3).

Intrinsic Fluorescence of the Native and Partially Denatured PGHS Isoforms—The fluorescence excitation and emission spectra of oPGHS-1 and hPGHS-2 were examined to determine appropriate wavelengths for monitoring denaturation. The two PGHS isoforms exhibited similar excitation spectra, with maxima near 282 nm; the emission maxima were at 337 nm for oPGHS-1 and 335 nm for hPGHS-2 (Fig. 2). The similar emission intensities in Fig. 2 from oPGHS-1, with nine tryptophan residues, and hPGHS-2, with six tryptophans, presumably reflects the higher concentration of hPGHS-2 used and the greater exposure of tryptophans to solvent in oPGHS-1 (Fig. 1). The spectral characteristics observed for hPGHS-2 are similar to those recently reported by Houtzager et al. (29). Incubation for 30 min in buffer containing 4 M GdmHCl shifted the emission maxima of both isoforms near 353 nm, with considerable decreases in intensity. These changes are consistent with unfolding of the proteins by the denaturant, with consequent exposure of normally buried tryptophan residues to the polar solvent. Excitation and emission wavelengths of 282 and 335–340 nm, respectively, were chosen to monitor GdmHCl-induced denaturation of the two isoforms. Over 90% of the fluorescence from hPGHS-2 under similar excitation and emission settings has been found to originate from tryptophan residues (29). This disproportionate contribution from tryptophan in a protein with 27 tyrosine residues and 6 tryptophan residues is counterintuitive, but it is in line with experience with other proteins (31). The fractional fluorescence contribution from tryptophan residues in oPGHS-1 is expected to be even greater, because it

\[ K = 2P_f(\beta_0/(1 - f_0)) \]  
(Eq. 2)
The emission intensity at 340 nm (excitation at 282 nm) was measured at the indicated times after incubation of oPGHS-1 apoenzyme (0.4 μM subunit) with 0 (open squares), 0.5 (filled squares), 1.5 (filled triangles), 3.0 (open triangles), or 5.0 M (filled circles) GdmHCl at room temperature. The curves for PGHS-1 and -2 run parallel, with the approximate values of K calculated from Equation 2 must be regarded as rough estimates. However, the curves for PGHS-1 and -2 run parallel, with the approximate K value for PGHS-2 greater than that for PGHS-1 throughout. This indicates that the hPGHS-2 structure was less stable than that of oPGHS-1 regardless of the denaturant level.

The reversibility of the denaturant-induced decreases in tryptophan fluorescence was examined for both isoforms (Fig. 4B). For enzyme preincubated with 6 M GdmHCl and subsequently diluted to lower denaturant levels, considerable fluorescence intensity was recovered after 30 min for both isoforms (Fig. 4B, solid symbols). However, the recovery of fluorescence was limited to slightly more than half the initial value for both oPGHS-1 or hPGHS-2, indicating that complete denaturation was not readily reversible for either isomere. When the PGHS isoforms were initially equilibrated with only 2 M GdmHCl, subsequent dilution was able to restore the fluorescence intensities to essentially the control levels (Fig. 4B, open symbols). This demonstrated that the early part of the denaturation process was completely reversible for both isoforms.

CD Changes in oPGHS-1 and hPGHS-2 upon Denaturation with GdmHCl—CD measurements were used as a second indicator of bulk structural changes during denaturation of the PGHS isoforms. In the native state, oPGHS-1 and hPGHS-2 had very similar CD spectra, with troughs at 209 nm and shoulders near 222 nm (Fig. 5). The α-helical contents predicted from these spectra were 38% for oPGHS-1 and 36% for hPGHS-2. These values are very close to the value of 38% helix calculated from secondary structure assignments based on the oPGHS-1 crystal structure (4); detailed secondary structure assignments were not reported for PGHS-2 (5, 6). Little organized β-sheet was reported for either PGHS-1 or PGHS-2 (4–6). Incubation for 30 min with 4 M GdmHCl greatly decreased the intensity of the CD trough for both isoforms (Fig. 5), indicating a considerable loss of helical structure (32). The CD intensity at 222 nm thus was chosen to monitor the helical content of both isoforms in subsequent denaturation experiments.

Dependence of oPGHS-1 and hPGHS-2 Secondary Structure on GdmHCl Concentration—Biphasic changes in the CD intensity at 222 nm with increasing denaturant were seen for both isoforms (Fig. 6). For oPGHS-1, the CD increased slightly by 0.5 M GdmHCl, then decreased monotonically with higher levels of denaturant, reaching minimal values near 6 M. For hPGHS-2, the initial increase was smaller, and the peak occurred at 0.2 M GdmHCl. The decrease in CD intensity for hPGHS-2 was larger than that for oPGHS-1 at all GdmHCl levels.
levels above 0.2 mM. Replots of the data in Fig. 6 to show approximate values of $K$ (calculated using Equation 2) as a function of the GdmHCl level produced parallel curves for PGHS-1 and -2 (not shown), much like the pattern obtained for the fluorescence data in the inset to Fig. 4A. The results from the CD measurements thus indicate that the secondary structure of hPGHS-2 was less stable than that of oPGHS-1, independent of the denaturant level. The overall patterns of CD changes with GdmHCl concentration (Fig. 6) were similar to those for the intrinsic fluorescence changes (Fig. 4A). This correlation between losses of secondary structure, monitored by CD, and increases in tryptophan exposure, monitored by fluorescence, support the use of the more convenient fluorescence measurements as an indicator of the overall folding status of the PGHS isoforms.

**Dependence of oPGHS-1 and hPGHS-2 Cyclooxygenase Activity on GdmHCl Concentration**—The cyclooxygenase activities were measured after incubation of concentrated or dilute solutions of each PGHS isoform with 0–3 M GdmHCl (Fig. 7A). For oPGHS-1, a monotonic loss of activity was seen for both levels of the enzyme, with an IC$_{50}$ value of 0.8 M GdmHCl for...
the concentrated enzyme (5 μM subunit) and 0.4 M GdmHCl for the dilute enzyme (0.5 μM subunit). Monotonic decreases in activity were also observed for both levels of hPGHS-2, with IC₅₀ values of 0.4 and 0.2 M GdmHCl for the concentrated (5 μM) and dilute (0.5 μM) enzyme solutions, respectively. Clearly, the hPGHS-2 cyclooxygenase activity was more easily disrupted by denaturant than the oPGHS-1 activity. Replots of the data in Fig. 7A to show approximate values of K (calculated using Equation 2) as a function of the GdmHCl level produced parallel curves for PGHS-1 and -2 (not shown), much like the pattern obtained for the fluorescence data in the "Experimental Procedures." Panel A, chromatograms for oPGHS-1 equilibrated at the indicated GdmHCl levels.

For an overall appraisal of the reversibility of the denaturant-induced loss of cyclooxygenase activity, the activity was measured after a 50-fold dilution of oPGHS-1 and hPGHS-2 preincubated with 0–6 M GdmHCl (Fig. 7B). Considerable cyclooxygenase activity was recovered from both isoforms exposed to the lower GdmHCl levels. However, no activity was recovered from oPGHS-1 preincubated with denaturant levels over 3 M, or from hPGHS-2 exposed to levels above 2 M, indicating that irreversible inactivation of both isoforms occurred at higher denaturant levels.

The reversibility of the activity losses in oPGHS-1 and hPGHS-2 exposed to lower levels of denaturant was examined in more detail (Fig. 7C). Here, each isoform was preincubated with sufficient GdmHCl to produce essentially complete inactivation (2.5 M for oPGHS-1, 1.5 M for hPGHS-2) and then equilibrated at a lower denaturant level before cyclooxygenase assay. Lowering the denaturant level resulted in a progressive recovery of activity in both isoforms, with essentially complete recovery of activity when the denaturant levels were reduced below 0.1 M. Thus, the inactivation of cyclooxygenase activity observed at lower GdmHCl levels is completely reversible in both PGHS isoforms. A larger fraction of the activity was recovered from oPGHS-1 than for hPGHS-2 at each GdmHCl level (Fig. 7C), consistent with the unfolding results in Fig. 7A, again indicating a higher active site structural stability in oPGHS-1.

**Dependence of oPGHS-1 and hPGHS-2 Hydrodynamic Properties on GdmHCl Concentration—**The effects of denaturant concentration on the hydrodynamic properties of the two PGHS isoforms were characterized by size exclusion chromatography (Figs. 8 and 9). Native oPGHS-1 and hPGHS-2 eluted in nearly symmetrical peaks with retention times of about 9.15 min, indicating the presence of a single species or multiple rapidly exchanging equilibrating species. However, the retention times continued to increase as the GdmHCl level increased above 3 M (Fig. 9A), with minima reached at 4 M for hPGHS-2 and near 6 M for oPGHS-1. Such an increase in the hydrodynamic size is consistent with complete unfolding of a monomeric species and has been observed with other proteins (36–38).

**Dependence of Subunit Cross-linking on GdmHCl Concentration—**The two isoforms underwent very similar changes in hydrodynamic properties as the denaturant level was raised (Fig. 9). oPGHS-1 was chosen to evaluate the relationship between the hydrodynamic changes and the oligomeric state. The effect of denaturant level on the proportion of oPGHS-1 in the dimeric state was assessed by examining the efficiency of monomer cross-linking by glutaraldehyde at two different protein concentrations. Electrophoretic results for cross-linking of 0.15 μM oPGHS-1 are shown in Fig. 10A. In the absence of denaturant, some of the protein migrated as a band with an apparent molecular mass of about 62 kDa, corresponding to the monomer. The remaining protein was in several ill-defined bands with apparent molecular masses of 150 kDa and above, presumably reflecting cross-linked dimers and higher oli-

**FIG. 8. Effect of GdmHCl on chromatographic profiles of oPGHS-1 and hPGHS-2.** The apoenzymes (2.5 μM subunit) were incubated for at least 30 min at room temperature in 0.1 M potassium phosphate, pH 7.2, containing 0.1% Tween 20 and 0–6 M GdmHCl. Aliquots (3 μl) were analyzed by size-exclusion chromatography on a column equilibrated with the same level of GdmHCl, as described under "Experimental Procedures." Panel A, chromatograms for oPGHS-1 equilibrated at the indicated GdmHCl levels. Panel B, chromatograms for hPGHS-2 equilibrated at the indicated GdmHCl levels.

The species with Stokes radii of 5.5 and 3.5 nm were not resolved, but the transition between the two was reflected in the changes of peak width at half-height (Fig. 9B). For both PGHS isoforms, the peak width increased at GdmHCl levels above 0.5 M, peaked at 1.25 M, and declined to a stable value above 2 M denaturant. Conversion from the 5.5-nm species to the 3.5-nm species thus occurred over the range of 0.5–2 M GdmHCl. The significant peak widening indicates the presence of both species at intermediate denaturant levels. Maximal peak width is expected when the amounts of the two species are equal, putting the midpoint of the transition near 1.25 M GdmHCl for both isoforms.

Further increases in GdmHCl level above 2 M did not change the peak width for either oPGHS-1 or hPGHS-2 (Fig. 9B), indicating the presence of a single species or multiple rapidly equilibrating species. However, the retention times continued to decrease as the GdmHCl level was raised above 2 M (Fig. 9A), with minima reached at 4 M for hPGHS-2 and near 6 M for oPGHS-1. Such an increase in the hydrodynamic size is consistent with complete unfolding of a monomeric species and has been observed with other proteins (36–38).

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gomers. As the denaturant level was increased, the proportion of protein in the monomer band increased, whereas that in the multimer bands decreased. The quantitative densitometric results from the gel in Fig. 10A are shown in Fig. 10B, along with the results from parallel incubations with a 10-fold higher protein level. For incubations with 0.15 M oPGHS-1, the transition from high to low cross-linking efficiency began at about 0.2 M GdmHCl and had an end point near 1.3 M GdmHCl, with a midpoint near 0.7 M GdmHCl. For incubations with the higher level of oPGHS-1, the start and end points of the transition were at 0.2 and 1.8 M GdmHCl, with a midpoint near 1.0 M GdmHCl. These GdmHCl ranges are comparable to those bracketing the transitions in PGHS-1 and -2 hydrodynamic properties (Fig. 9B).

A transition in efficiency of subunit cross-linking by glutaraldehyde was found at a much higher GdmHCl level, around 5 M, for transthyretin (39), showing that the oPGHS-1 cross-linking transitions are not due to effects of the denaturant on glutaraldehyde reactivity. The cross-linking efficiency in the absence of denaturant was little affected by the 10-fold change in protein concentration, with 47% as monomer with 0.15 μM enzyme and 41% as monomer with 1.5 μM enzyme. This indicates that the observed loss of monomer is mostly due to cross-linking within dimers, rather than between dimers or between dissociated monomers, because the latter two reactions require bimolecular collisions and are consequently very sensitive to protein level. Cross-linking of monomers within dimers thus was less efficient with increasing denaturant. The straightforward interpretation is that the denaturant increases dissociation of dimers to monomers. This interpretation is supported by the observation that increasing the protein level, which shifts the dimer ⇔ monomer equilibrium in favor of the dimer, raises the midpoint GdmHCl level for the transition in cross-linking efficiency (Fig. 10B). Overall, the cross-linking results provide independent evidence that the very similar transitions in hydrodynamic properties observed for PGHS-1 and -2 at 0.5–2 M GdmHCl (Fig. 9B) reflect dimer ⇔ monomer transitions.

Comparison of Cyclooxygenase and Peroxidase Active Site Stabilities in oPGHS-1 and hPGHS-2—The stabilities of the native structures of the active sites in oPGHS-1 and hPGHS-2 were further assessed by monitoring decreases in peroxidase activity and cyclooxygenase-inhibitor complex formation after equilibration of the proteins with various levels of GdmHCl; the results are shown in Fig. 11. Inhibition of the peroxidase activity of both isoforms was observed, even at the lowest levels...
of denaturant tested, and progressed as the levels were increased (Fig. 11A). The IC_{50} values for peroxidase activity were 0.4 and 0.2 M GdmHCl for oPGHS-1 and hPGHS-2, respectively, showing that the PGHS-2 activity was more easily disrupted than that of PGHS-1. The inhibitory effects on the peroxidase activities of both isoforms, with more extensive loss of activity for hPGHS-2 than in oPGHS-1.

Comparison of Stabilities of Cyclooxygenase Activities in the Two Human PGHS Isoforms—Partially purified samples of hPGHS-1 and hPGHS-2, with comparable concentrations of the recombinant proteins, were equilibrated with various GdmHCl levels before assay of cyclooxygenase activity (Fig. 12). A monotonic loss of activity with increasing GdmHCl was seen with both isoforms, with more extensive loss of activity for hPGHS-2 than for hPGHS-1 at each denaturant level below 2 M. The IC_{50} values for hPGHS-1 and hPGHS-2 were near 0.5 M and 0.2 M GdmHCl, respectively. The results of this comparison of hPGHS-1 with hPGHS-2 (Fig. 12) were similar with those in the comparison of oPGHS-1 with hPGHS-2 (Fig. 7A). Thus, the cyclooxygenase activities of both hPGHS-1 and oPGHS-1 are more stable than the hPGHS-2 activity.

Flurbiprofen and indomethacin are tight-binding, stoichiometric cyclooxygenase inhibitors for purified PGHS-1 and -2; both compete for the arachidonate binding site (2, 4, 27, 28). The action of these agents involves an initial reversible binding, followed by a time-dependent conversion of the initial enzyme-inhibitor complex to a much higher affinity complex (27, 40, 41). Complex formation can be monitored by changes in the intrinsic fluorescence of the proteins (see Ref. 29 and “Experimental Procedures”). The ability to form the tight complex with these inhibitors thus furnishes a convenient way to assess the overall structural integrity of the cyclooxygenase active site. Preincubation of PGHS-1 and -2 with low levels of GdmHCl led to loss of their ability to form complexes with indomethacin and flurbiprofen (Fig. 11B). The IC_{50} values for indomethacin binding were 0.45 and 0.25 M GdmHCl for oPGHS-1 and hPGHS-2, respectively. For flurbiprofen binding, the IC_{50} values were 0.45 and 0.2 M GdmHCl for oPGHS-1 and hPGHS-2, respectively. Thus, results with both probes indicated that the cyclooxygenase active site in hPGHS-2 was considerably more sensitive to disruption than the site in oPGHS-1. For each isoform, the effects of denaturant on inhibitor complex formation were almost the same as those on peroxidase and cyclooxygenase activity (Fig. 11, A and B). Replots of the data in Fig. 11 to show approximate values of K (calculated using Equation 2) as a function of the GdmHCl level produced parallel curves for PGHS-1 and -2 (not shown), much like the pattern obtained for the fluorescence data in the inset to Fig. 4A. Thus, the results in Fig. 11 indicate that both the cyclooxygenase and peroxidase active sites were less stable in hPGHS-2 than in oPGHS-1.
DISCUSSION

Previous hydrodynamic and cross-linking studies established that both oPGHS-1 and hPGHS-2 are dimers after solubilization with Tween 20 (33, 34, 42). The stabilizing effects of quaternary structure in oligomeric proteins increase the potential complexity of the denaturation process (15). Nevertheless, in many oligomers dissociation destabilizes the monomers to such an extent that these systems can be considered as two-state equilibria between native oligomers and completely unfolded monomers, permitting detailed thermodynamic analyses (15). With both oPGHS-1 and hPGHS-2, however, the complex patterns observed for loss of higher order structure with increasing denaturant level (Figs. 4, 6, 8, and 9) show that the two-state model does not completely describe the unfolding process for either PGHS isoform.

For both isoforms, the major loss of overall secondary and tertiary structure occurs above 0.5 M GdmHCl, with its onset overlapping with the transition from dimers to monomers monitored by hydrodynamic and cross-linking properties (Figs. 8–10). It is important to emphasize that the dimer dissociation expected from the protein concentration-dependence of the fluorescence and activity denaturation curves (Figs. 4 and 7) is corroborated by the hydrodynamic and cross-linking observations (Figs. 8–10). Dissociation does not totally disrupt the monomer structure in either isoform, because the monomers retain considerable structure and appear to be relatively compact until the denaturant level is raised considerably higher (Figs. 4A, 6, and 9). The later part of the decline in secondary and tertiary structure observed in fluorescence and CD measurements (Figs. 4A and 6) thus reflects the unfolding of these compact monomeric intermediates. Several examples are known of dimeric proteins dissociating to relatively stable monomers with considerable secondary and tertiary structure (36–38, 43).

It is interesting that the loss of quaternary structure and activity is completely reversible for both isoforms, whereas unfolding of the monomers is not readily reversible (Figs. 4B, 7B, and 7C). This suggests that disordered PGHS monomers may not be able to properly fold to a compact intermediate by themselves. Once the compact monomer fold is reached, however, it seems likely that assembly into dimers can proceed spontaneously. The observed recovery of activity from PGHS-1 and -2 treated with GdmHCl levels sufficient to cause dissociation of the dimers into monomers (Figs. 7A–10) raises the possibility of isolating undamaged monomers by judicious denaturant treatment. Such monomers would be very useful in evaluating the nature and functional consequences of monomer-monomer interactions in homodimers and heterodimers of the two PGHS isoforms.

Disruption of cyclooxygenase activity by GdmHCl was also sensitive to the protein concentration for both isoforms (Fig. 7A), demonstrating that loss of activity is coupled to dissociation of the dimers. However, significant loss of activity occurred at GdmHCl levels which produced little change in the fluorescence signal or in the hydrodynamic and cross-linking behavior (Figs. 4A, 7A, and 9–11). This indicates some localized loss of active site structure occurs without disruption of the overall secondary or tertiary structure in the subunits, or of the interactions between the subunits. Thus, dimeric structure is necessary, but not sufficient, for activity in both PGHS isoforms. A simple model consistent with the present data has two intermediates between native dimers (M-M) and completely unfolded monomers (U) (Reaction 1).

\[
\begin{align*}
K_1 & : M-M \leftrightarrow M^* \cdot M^* \\
K_2 & : M^* \cdot M^* \leftrightarrow 2M \\
K_3 & : 2M \leftrightarrow 2U
\end{align*}
\]

**REACTION 1**

The complexity of the denaturation process and the inability of denatured monomers to return to native dimers makes it difficult to calculate thermodynamic parameters for either isoform. However, the midpoint for dissociation to monomers was near 1.25 M GdmHCl for both oPGHS-1 and hPGHS-2 (Fig. 9), indicating that the two isoforms have similar \( K_s \) values. Because of this, if PGHS-1 and PGHS-2 are compared at the same protein concentration, the relative stabilities of their active site structure (i.e. the M-M ↔ M*·M* process in Reaction 1) can be gauged from their sensitivities to inactivation by denaturant. To simplify this comparison, the \( IC_{50} \) values for cyclooxygenase, peroxidase, and cyclooxygenase inhibitor binding activities calculated from the data in Figs. 7A, 11, and 12 are presented in Table I. It should be noted that these \( IC_{50} \) values are not equivalent to the \( C_{50} \), GdmHCl or midpoint \( \Delta G \) values used in reference to thermodynamically better defined systems (15).

Two major points emerge from the data in Table I. First, the separate measurements of peroxidase and cyclooxygenase active site stability produce convergent values. The cyclooxygenase activity of both PGHS isoforms requires initiation via peroxidase intermediates (17, 44, 54). Because of this mechanistic linkage, disruption of the peroxidase site would be expected to lead to indirect loss of cyclooxygenase activity. Thus, it is not surprising that the cyclooxygenase and peroxidase activities were lost in concert as the denaturant concentration was increased (Figs. 7A and 11A). However, the binding of cyclooxygenase inhibitors, which provides a direct assessment of cyclooxygenase site integrity, displayed about the same sensitivity to denaturant as the enzymatic activities in both isoforms (Fig. 11). This indicates that the folding stability at the peroxidase site is comparable to that at the cyclooxygenase site. Although distinct from one another, the two active sites are parts of the same folded domain in both isoforms (4–6), and it may be that disruption of one site is physically coupled to disruption of the other.

The second pattern evident from Table I is that all measurements of active site function indicate that the PGHS-1 active sites are considerably more stable than those in PGHS-2. It remains to be seen whether the lower stability of the PGHS-2 active sites to denaturant reflects a higher flexibility in the native state, as shown experimentally by measurement of con-
formational mobility for several other enzymes (45). Higher flexibility in the active site might well be a major factor in the ability of PGHS-2 to accommodate the relatively bulky isoform-2 specific inhibitors, and it is consistent with the more expansive cyclooxygenase site observed in crystal structures of PGHS-2 with bound inhibitors (5, 6). A lower cyclooxygenase active site flexibility in PGHS-1 would also help explain why acetylation of the active site serine by aspirin completely blocks fatty acid oxygenase activity in that isoform, whereas the steric impediment of the acetyl group is tolerated in the PGHS-2 cyclooxygenase active site (8, 9, 46).

The two amino acid differences between the isoforms in the immediate cyclooxygenase pocket, at positions 499 and 509 in hPGHS-2, have been shown to affect the interactions with specific inhibitors (11–13) and thus may contribute to the difference in active site stability between the isoforms. These substitutions are subtle, but addition of a single methylene group can significantly alter a protein's folding stability (47). The peroxidase site residues are less well conserved between the two PGHS isoforms, and so it is more difficult to postulate a structural basis for the difference between the isoforms in the stability at that site. Curiously, the loop near Arg-277 in PGHS-1 (Pro-263 in PGHS-2), which lies near the peroxidase site, is protease-sensitive in PGHS-1 but protease-resistant in PGHS-2 (48), suggesting that this structural element is more stable in PGHS-2.

The active site structures of both isoforms are more easily destabilized than the overall folding, with cyclooxygenase and peroxidase activities dropping significantly at GdmHCl levels which caused little change in fluorescence or CD intensities (Figs. 4, 6, 7, and 11). Thus, the enzymatic activities serve as sensitive indicators of the early stages of unfolding. The fact that the levels of non-ionic detergents commonly used to maintain the isoforms in solubilized form do not lead to loss of activity indicates that these detergents do not induce a significant degree of unfolding. The PGHS-1 and -2 crystallographic data were obtained using enzyme solubilized with such non-ionic detergents (4–6), further demonstrating that the levels of detergents generally used with the enzymes are not structurally disruptive. It is possible that the unfolding equilibria are perturbed by changes in detergent level. This would not change the conclusions about the relative stability of the two isoforms drawn in the present study, because a fixed, low concentration of Tween 20 was used for all experiments.

As mentioned above, the hydrodynamic results indicate that dissociation of dimers to monomers occurs over similar denaturant levels for the two PGHS isoforms, indicating little difference in the second step of the denaturation scheme in Reaction 1. Several amino acid differences between the isoforms have been noted in the subunit-subunit interface (5), but these apparently are not enough to have a large impact on the strength of the interaction between the subunits. The interface area in the PGHS-2 dimer, 2700 Å² (5), is well below the value of about 4000 Å² expected for a subunit of 70 kDa (49). Assuming that the strength of quaternary interactions is proportional to the interfaceal area, this would predict that the quaternary interactions in PGHS-2 are weaker than in the average dimer. This prediction is consistent with observations indicating dissociation of both PGHS isoforms without complete destabilization of the monomers (Figs. 4, 6, and 9). Fluorescence, CD, and hydrodynamic changes at GdmHCl levels above 2 M were more pronounced for hPGHS-2 than for oPGHS-1 (Figs. 4A, 6, and 9). Both isoforms are predominantly in a monomeric state at these denaturant levels (Fig. 9), so the third step in the denaturation process, the unfolding of compact monomers (Reaction 1), appears to occur more readily in hPGHS-2 than in oPGHS-1. This would suggest a lower stability in PGHS-2 than in PGHS-1 of the secondary and tertiary structure in isolated monomers. The situation is complicated, however, by the fact that loss of secondary and tertiary structure begins well before complete conversion to monomers, and the structural disruption is sensitive to the protein concentration (Figs. 4A, 6, and 9). This indicates that considerable changes in subunit folding accompany dissociation of the dimers, with the fraction of subunit unfolding occurring during and after dissociation difficult to estimate. In any case, the overall stability of secondary and tertiary structure is considerably higher in oPGHS-1 than in hPGHS-2.

The sensitivity of the denaturation process to the protein concentration for both PGHS-1 and PGHS-2 (Figs. 4A and 7A) raises the possibility that the differences in denaturant susceptibilities between the isoforms are due to different amounts of “silent,” inactive PGHS protein in the electrophoretically pure oPGHS-1 and hPGHS-2 preparations. One way to assess the presence of such inactive protein is to monitor the stoichiometry of heme cofactor needed to restore full cyclooxygenase activity to the purified apoenzyme. For oPGHS-1, titrations with heme had an average end point of about 0.7 heme/subunit (50–52); similar titration of recombinant hPGHS-2 expressed in the baculovirus system produced an end point near 0.9 heme/subunit (34). The difference between the end points indicates a potential difference of about 30% in the proportion of inactive protein. This potential difference is unable to account for more than a small fraction of the observed shifts between the PGHS-1 and -2 curves, which were roughly equivalent to a 10-fold change in protein level (Figs. 4 and 7). As noted earlier, the oPGHS-1 helical content calculated from CD measurements is indistinguishable from that calculated from the crystal structure, indicating that the secondary structure was largely intact even if some of the preparation was unable to bind heme.

The present studies used detergent-solubilized preparations of the PGHS isoforms. Both isoforms are intrinsic membrane proteins and are thought to be bound to the endoplasmic reticulum membrane via amphipathic helical segments on one face of the dimeric structures (4–6). The structural changes that occur upon solubilization are not known, but they do not grossly affect the enzymatic activities or the responsiveness to inhibitors. In addition, the putative membrane anchor comprises a relatively small, poorly conserved portion of the PGHS proteins, well removed from the interface between the subunits; the bulk of the proteins are globular (4–6). All of this makes it likely that the native lipid membrane has little influence on the folding stability of either PGHS isoform, and that the folding stabilities of the isolated, solubilized proteins will be similar to those of the cellular enzymes.

oPGHS-1 was used in the present studies because the ovine enzyme can readily be purified in the quantities required for biophysical studies. Recombinant hPGHS-1 can be expressed in the baculoviral system, but we have found the yields to be considerably lower than those for hPGHS-2. The human and ovine PGHS-1 amino acid sequences have 91% identity and 96% similarity; conservation in the cyclooxygenase active site is essentially complete (53). There are very few non-conservative residue substitutions between human and ovine PGHS-1 at the interface between the subunits, suggesting a similar dimer stability in the two enzymes. Human and ovine PGHS-1 also have quite similar protease susceptibilities (48), and the cyclooxygenase activity of hPGHS-1, like that of oPGHS-1, is more resistant to denaturation than the hPGHS-2 activity (Figs. 7A and 11). These consistently similar properties of the human and ovine enzymes indicate that the conclusions drawn
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from the present studies with oPGHS-1 will be valid for hPGHS-1 as well.

In summary, results from these denaturation studies show that the two PGHS isoforms have similar quaternary structure stabilities, but differ markedly in their secondary and tertiary structural stabilities, with PGHS-2 having a lower folding stability both in the cyclooxygenase and peroxidase active sites and in the subunit as a whole. This difference in the stability of the folding of the two PGHS isoforms may well be related to the observed differences in their functional interactions with substrates, activators, and inhibitors.

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Comparison of Structural Stabilities of Prostaglandin H Synthase-1 and -2
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