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Characterization of the Unfolding Process of Lipocalin-type Prostaglandin D Synthase*

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Takashi Inui‡§¶, Tadayasu Ohkubo‖, Maiko Emi§, Daisuke Irikura‡§, Osamu Hayaishi§, and Yoshihiro Urade‡§‡‡**

From the ‡Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation and §Department of Molecular Behavioral Biology, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565-0874, Japan and the ‖Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

We found that low concentrations of guanidine hydrochloride (GdnHCl, <0.75 M) or urea (<1.5 M) enhanced the enzyme activity of lipocalin-type prostaglandin (PG) D synthase (L-PGDS) maximally 2.5- and 1.6-fold at 0.5 M GdnHCl and 1 M urea, respectively. The catalytic constants in the absence of denaturant and in the presence of 0.5 M GdnHCl or 1 M urea were 22, 57, and 30 min^{-1}, respectively, and the K_m values for the substrate, PGH_2, were 2.8, 8.3, and 2.3 μM, respectively, suggesting that the increase in the catalytic constant was mainly responsible for the activation of L-PGDS. The intensity of the circular dichroism (CD) spectrum at 218 nm, reflecting the β-sheet content, was also increased by either denaturant in a concentration-dependent manner, with the maximum at 0.5 M GdnHCl or 1 M urea. By plotting the enzyme activities against the ellipticities at 218 nm of the CD spectra of L-PGDS in the presence or absence of GdnHCl or urea, we found two states in the reversible folding process of L-PGDS: one is an activity-enhanced state and the other, an inactive state. The NMR analysis of L-PGDS revealed that the hydrogen-bond network was reorganized to be increased in the activity-enhanced state formed in the presence of 0.5 M GdnHCl or 1 M urea and to be decreased but still remain in the inactive intermediate observed in the presence of 2 M GdnHCl or 4 M urea. Furthermore, binding of the non-substrate ligands, bilirubin or 13-*cis*-retinal, to L-PGDS changed from a multistate mode in the native form of L-PGDS to a simple two-state mode in the activity-enhanced form, as monitored by CD spectra of the bound ligands. Therefore, L-PGDS is a unique protein whose enzyme activity and ligand-binding property are biphasically altered during the unfolding process by denaturants.

Lipocalin-type prostaglandin (PG)[1] D synthase (L-PGDS, prostaglandin-H_2 D-isomerase, EC 5.3.99.2) is abundantly expressed in the central nervous system and male genitals of various mammals and in the human heart (1, 2). In these tissues, L-PGDS catalyzes the isomerization of PGH_2, a common precursor of various prostanoids, to PGD_2 in the presence of sulfhydryl compounds (3). PGD_2 acts as a neuromodulator in the central nervous system, where it induces sleep and regulates body temperature, luteinizing hormone release, and pain responses (1, 2). In the peripheral tissues, PGD_2 functions as a mediator of allergy and inflammatory responses (2, 4). Sequence analyses and a homology search in data bases of protein primary structure have revealed that L-PGDS is a member of the lipocalin superfamily (1, 5), which is composed of various secretory lipid-transporter proteins, such as β-lactoglobulin, retinol-binding protein, major urinary protein, and bilin-binding protein (6). Lipocalin is a family of diverse proteins that normally serve for the storage or transport of physiologically important lipophilic ligands (6–8). L-PGDS also has the ability to bind retinoids, bile pigments such as bilirubin and biliverdin, and thyroid hormones (9, 10). Therefore, L-PGDS is a unique dual functional protein in the lipocalin family, acting as a PGD_2-synthesizing enzyme and also as an extracellular transporter protein for lipophilic ligands.

Despite their low mutual sequence homology, members of the lipocalin family share a conserved barrel of 8 antiparallel β-strands as their central folding motif; and the large cup-shaped hydrophobic cavity within the β-barrel and a loop scaffold at its entrance are well adapted to the task of ligand binding (11–15). The preliminary crystal structure analysis revealed that the tertiary structure of mouse recombinant L-PGDS also showed an 8-stranded β-barrel structure, and one free SH group because of Cys^{65} faced the inside of the hydrophobic cavity of the barrel structure (1). The enzyme activity of L-PGDS disappeared completely by chemical modification or replacement of the Cys^{65} residue with serine or alanine by site-directed mutagenesis (16). Therefore, Cys^{65} is considered to play an important role as an active center for the catalytic function of L-PGDS.

We recently found that the L-PGDS activity was enhanced in the presence of low concentrations of guanidine hydrochloride (GdnHCl) (17). In most cases, an enzyme is inactivated by denaturants at concentrations lower than those required for the complete unfolding (18). However, a few enzymes have been reported to be activated instead. For example, adenylate kinase

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¶ Present address: Dept. of Food and Nutrition, Tsu City College, 157, Ishinden, Tsu City, Mie 514-0112, Japan.

** Present address: Dept. of Pharmacology, Ehime University School of Medicine, Shigenobu-cho, Onsen-gun, Ehime 791-0295, Japan.

‡‡ To whom correspondence should be addressed: Dept. of Molecular Behavioral Biology, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565-0874, Japan. Tel.: 81-6-6872-4851; Fax: 81-6-6872-2841; E-mail: uradey@obi.or.jp.

[1] The abbreviations used are: PG, prostaglandin; L-PGDS, lipocalin-type prostaglandin D synthase; GdnHCl, guanidine hydrochloride; k_{cat}, catalytic constant; K_d, dissociation constant; CSF, cerebrospinal fluid; SAH, aneurysmal subarachnoid hemorrhage; deg, degree.

was reported to be activated in the presence of urea up to 1 M or up to 0.25 M GdnHCl (19). Dihydrofolate reductase also showed enhanced enzyme activity in the presence of urea up to 7 M (20, 21). By measuring the change in the fluorescence of 8-anilino-1-naphthalenesulfonic acid binding to adenylate kinase during denaturation and the activity change in dihydrofolate reductase during trypsin digestion in the presence of GdnHCl or urea, both groups of investigators suggested that the enzyme activation was caused by increased flexibility of the conformation at the enzyme active site. On the other hand, the mechanism of L-PGDS activation by denaturants has remained unclear.

In this study, we investigated the unfolding process of L-PGDS in the presence of GdnHCl and urea by CD and NMR analyses combined with measurements of its synthase and ligand-binding activities. We found that two states were formed during the unfolding process, an activity-enhanced state at a low concentration of denaturants and an inactive intermediate at a higher concentration of denaturants. The enzyme activity was in good correlation with the amount of secondary structure, especially the amount of β-sheet structure. The ligand binding mode was also changed from a multistate mode for the native form of L-PGDS in the absence of denaturants to a two-state mode for the activity-enhanced form. The inactive intermediate still retained a secondary structure to maintain the folded core despite nearly complete loss of activity. Based on our findings, we propose a four-state equilibrium folding model for L-PGDS composed of the activity-enhanced and inactive states.

EXPERIMENTAL PROCEDURES

Materials—GdnHCl, urea, SDS, and dithiothreitol were purchased from WAKO (Tokyo, Japan). Thrombin, bilirubin, and 13-*cis*-retinal were obtained from Sigma. All other chemicals were of analytical grade.

Expression of Recombinant Mouse L-PGDS—The full-length cDNA for mouse L-PGDS, which is composed of 189 amino acid residues (GenBank™ accession number X89222 (22)), was ligated into the *Bam*HI-*Eco*RI sites of the expression vector pGEX-2T plasmid (Amersham Biosciences). The N-terminal 22-amino acid residues of the signal peptide were deleted, and the C89A/C186A-, W43F-, and W54F-substituted recombinant enzymes were expressed in *Escherichia coli* DH5α (TOYOBO, Tokyo, Japan). Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). The DNA sequence were confirmed with a LI-COR model 4000L automated DNA sequencer (LI-COR Inc., Lincoln, NE) after cycle sequencing with a SequiTherm cycle sequencing kit (Epicentre Technologies, Madison, WI). The recombinant enzymes retained enzymatic activity comparable with that of the wild-type L-PGDS and is stable for long-term use. The mutated L-PGDS was expressed as a glutathione transferase fusion protein. The fusion protein was bound to glutathione-Sepharose 4B (Amersham Biosciences) and incubated with thrombin (100 units/100 μl) to release the L-PGDS. The recombinant protein was further purified to apparent homogeneity by Superdex 75 and Mono-Q column chromatographies. The purified protein was dialyzed against 5 mM Tris/HCl (pH 8.0).

Purification of Human L-PGDS in Cerebrospinal Fluid (CSF) after Aneurysmal Subarachnoid Hemorrhage (SAH)—Patients with aneurysmal SAH, who were consecutively admitted to the Department of Neurosurgery, Nagoya City University Hospital, were treated with intravascular embolization of the aneurysmal dome by using detachable coils or surgically clipping of the dome within 24 h after the initial SAH attack. After the treatment of the aneurysms, a silicon drainage tube was placed in the lumbar CSF space for continuous draining of the subarachnoid clots into the CSF. Approximately 2 months after SAH, the normal CSF of patients (which does not contain blood) who developed communicating hydrocephalus was collected over 3 days. Informed consent was obtained from all patients to perform the lumbar puncture, which was part of the diagnostic workup. Human L-PGDS was purified from the CSF by immunoaffinity chromatography with the monoclonal antibody 1B7-conjugated column, as reported previously (23). Human L-PGDS was further purified to apparent homogeneity by Superdex 75 column chromatography (Amersham Biosciences).

Enzyme Assays—The L-PGDS activity was measured by incubating the enzyme at 25 °C for 1 min with [1-^{14}C]PGH_2 (final concentration of 40 μM) in 50 μl of 0.1 M Tris/HCl (pH 8.0) containing 1 mg/ml IgG and 1 mM dithiothreitol (16). [1-^{14}C]PGH_2 was prepared from [1-^{14}C]arachidonic acid (2.20 GBq/mmol, PerkinElmer Life Sciences) as described previously (3).

CD Measurements—The CD spectrum of L-PGDS was measured with a spectropolarimeter model J-700 (Jasco, Tokyo, Japan). The temperature of the solution in the cuvette was controlled at 4.0 ± 0.5 or 25.0 ± 0.5 °C by circulating water. The CD spectra were essentially the same between these two temperatures. The path length of the optical quartz cuvette was 1.0 mm for far-UV range CD measurements at 200 to 250 nm, and 10 mm for near-UV to visible range CD measurements at 250 to 600 nm. The protein solutions contained enzyme at 200 μg/ml, 5 mM Tris/HCl (pH 8.0), and the appropriate amount of denaturant, bilirubin, or 13-*cis*-retinal. The data were expressed as molar residue ellipticity (θ).

Fluorescence Quenching Assays—Various concentrations of bilirubin or 13-*cis*-retinal (10 μl) were added to L-PGDS in 990 μl of 5 mM Tris/HCl (pH 8.0) to give a final concentration of 1.5 μM in the presence or absence of denaturant. Bilirubin was dissolved in Me_2SO to give a 2 mM stock solution, and 13-*cis*-retinal in ethanol to give a stock solution of 1 mM. The concentrations of hydrophobic ligands were determined spectroscopically based on their respective molar absorption coefficients, *i.e.* ϵ_{453} in chloroform for bilirubin = 61,700 M^{-1} cm^{-1} (24) and ϵ_{383} for 13-*cis*-retinal = 42,800 M^{-1} cm^{-1} (25). After incubation at 25 °C for 60 min, the intrinsic tryptophan fluorescence was measured by an FP-750 spectrofluorometer (Jasco) with an excitation wavelength at 282 nm and an emission wavelength at 338 nm. The quenching of tryptophan fluorescence caused by nonspecific interactions with each ligand was corrected with 1.5 μM *N*-acetyl-L-tryptophanamide. The dissociation constant (K_d) value for binding between ligand and L-PGDS was calculated by the method as described previously (26).

NMR Spectroscopy—Uniformly ^{15}N-labeled protein was obtained by growing the transformed *E. coli* in M9 minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source. Samples for NMR spectroscopy were dissolved in 90% H_2O, 10% D_2O-containing 50 mM sodium phosphate, pH 8.0, and the protein concentrations were set at 2 mg/ml in 250 μl in a 5-mm microcell NMR tube (Shigemi Inc., Tokyo, Japan). ^{15}N-^{1}H heteronuclear multiple quantum coherence spectra (27) were measured with a Varian Unity Inova 500 spectrometer (Varian Instruments, Palo Alto, CA) equipped with a triple resonance probe. Water suppression was achieved by solvent presaturation. Under the condition of pH 8.0, the time constant for intrinsic amide hydrogen exchange was about 10 ms, and so presaturation of the water signal obliterated the signals of rapidly exchanging protons in unstructured parts of the protein (28). One-dimensional ^{1}H NMR spectra of L-PGDS were obtained in the absence or presence of 0.5, 2, or 6 M GdnHCl. Considering the protein solubility during the GdnHCl titration, we set the protein concentrations at 250 μg/ml. The temperature for NMR experiments was 30 °C. Phase-shifted sine-bell window functions, solvent-suppression filter on the time-domain data, and zero filling were applied before Fourier transformation. ^{1}H chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate and were measured with respect to acetone as an internal standard. The ^{15}N chemical shifts were measured relative to an external standard, NH_4Cl.

Statistical Analysis—Data were expressed as the mean ± S.E. The statistical significance between the control and the experimental group was assessed by Student's t test. $p < 0.05$ was considered to be significant.

RESULTS

Changes in Enzyme Activity and Secondary Structure of L-PGDS Induced by Denaturants—We investigated first the effects of denaturants, such as GdnHCl, urea, and SDS, on the enzyme activity of the C89A/C186A-substituted recombinant mouse L-PGDS. In the presence of 0.10–0.75 M GdnHCl or 0.25–1.50 M urea, the PGDS activity was significantly enhanced (Fig. 1*A*). At 0.5 M GdnHCl and 1 M urea, the enzyme activity reached its maximum of 2.5 ± 0.1 and 1.6 ± 0.1 μmol min^{-1} mg^{-1}, respectively, which was 2.5- and 1.6-fold, respectively, higher than the activity in the absence of denaturants (1.0 ± 0.1 μmol min^{-1} mg^{-1}). The enzyme activity was reduced above 0.5 M GdnHCl or 1 M urea in a concentration-dependent manner and became less than 5% of the control at 2 M GdnHCl or 4 M urea. Furthermore, the enzyme activity was completely

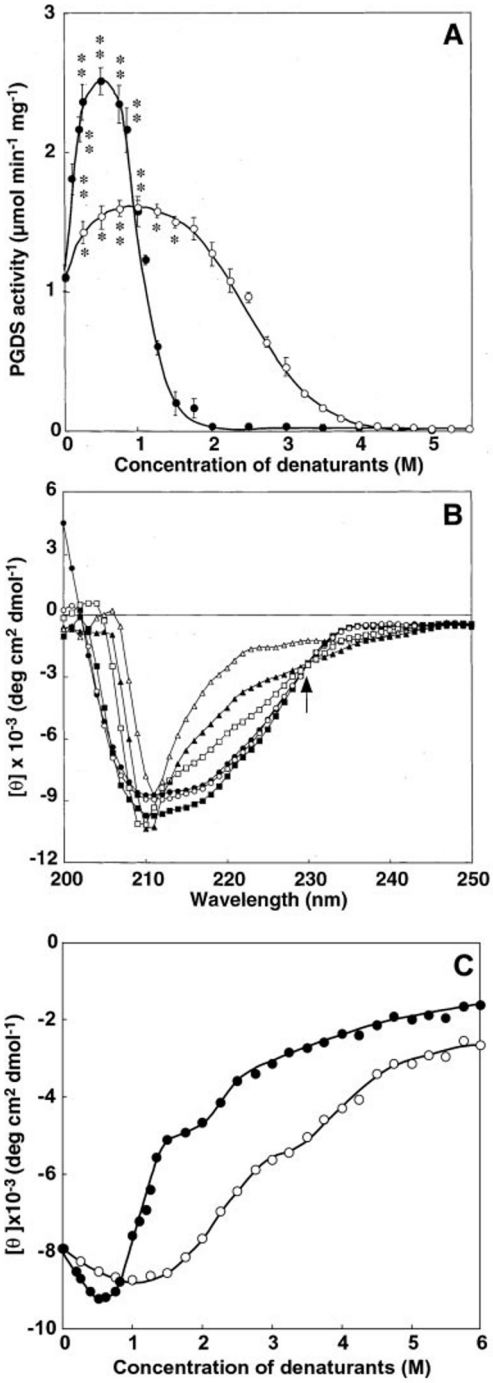

FIG. 1. **Changes in enzyme activity and secondary structure of L-PGDS induced by denaturants.** *A*, the *curves* show the changes in the enzyme activity of L-PGDS in the presence or absence of various concentrations of urea (○) or GdnHCl (●). The reaction was carried out at 25 °C for 1 min in the presence of 40 μM PGH_2 and 1 mM dithiothreitol, pH 8.0. Data are expressed as the mean ± S.E. of three independent experiments. Significant difference was based on Student's *t* test, **, $p < 0.01$; *, $p < 0.05$ *versus* in the absence of denaturants. *B*, CD spectra of L-PGDS in the far-UV region. The CD spectra of L-PGDS (200 μg/ml) were obtained at 4 °C, pH 8.0, in the absence (●) or presence of 0.25 (○), 1 (■), 2.5 (□), 4 (▲), or 6 (△) M urea. An *arrow* shows the isosbestic point. *C*, transition curves of L-PGDS unfolding induced by denaturants. The *curves* show the changes in the CD ellipticities (θ) at the wavelength of 218 nm, reflecting the content of β-sheet structure of L-PGDS, in the presence or absence of various concentrations of urea (○) or GdnHCl (●).

recovered by the depletion of denaturants (data not shown), showing the reversibility of the denaturant effect on folding. On the other hand, the enzyme activity of L-PGDS was reduced by SDS in a concentration-dependent manner without any enhancement of the enzyme activity and reached less than 5% at 0.3% SDS (data not shown). These results show that the L-PGDS activity was enhanced by low concentrations of urea or GdnHCl, but not by SDS.

TABLE I

Kinetic parameters and binding affinities of L-PGDS for lipophilic small ligands in the absence or presence of denaturants

The kinetic properties of L-PGDS in the absence or presence of denaturants were calculated. The K_d values were estimated by the fluorescence quenching data according to Levine (26). Data are expressed as the mean of three independent experiments.

Denaturant	K_m	k_{cat}	K_d for bilirubin	K_d for 13-*cis*-retinal
	μM	min^{-1}	nM	μM
(−)	2.8	22	110	1.0
0.5 M GdnHCl	8.3	57	450	1.2
1 M Urea	2.3	30	120	1.1

The PGDS activity of human L-PGDS purified from CSF after SAH also was significantly enhanced in the presence of 0.1–1 M GdnHCl or 0.25–2 M urea. At 0.5 M GdnHCl and 1 M urea, the enzyme activity reached its maximum, which was 3- and 1.7-fold, respectively, higher than the activity in the absence of denaturants.[2] The enzyme activity was reduced above 0.5 M GdnHCl or 1 M urea in a concentration-dependent manner and became less than 5% of the control at 3 M GdnHCl or 4 M urea. These results showed that the activation of L-PGDS occurred in the presence of low concentrations of denaturants not only on the recombinant protein but on the native one.

Next we determined the kinetic properties of the recombinant mouse L-PGDS in the absence or presence of denaturants (Table I). In the absence of denaturants, the apparent K_m for PGH_2 was 2.8 μM, and the catalytic constant (k_{cat}) was calculated to be 22 min^{-1}. In the presence of 0.5 M GdnHCl, the K_m was 8.3 μM and k_{cat} was 57 min^{-1} and in the presence of 1 M urea, the K_m dropped to 2.3 μM and k_{cat} rose to 30 min^{-1}. These results suggest that the enhancement of L-PGDS activity in the presence of the low concentrations of denaturants was mainly because of the increase in the catalytic constant.

The conformational changes in the recombinant mouse L-PGDS in the presence of denaturants were investigated by measuring the CD spectra. Fig. 1*B* shows the far-UV equilibrium CD spectra of L-PGDS in the absence or presence of 0.25, 1, 2.5, 4, and 6 M urea. The far-UV CD in the native state of L-PGDS showed a spectrum with an abundance of β-sheets (*closed circle*). By the treatment with 0.25 (*open circle*) or 1 (*closed square*) M urea, the absolute CD intensity was increased over a range of wavelengths from 206 to 230 nm, showing the augmentation of the secondary structure of L-PGDS. The secondary structure gradually disappeared by an increase in the urea concentration (2.5 M, *open square*; 4 M, *closed triangle*) and was completely lost at 6 M urea (*open triangle*). A tight isosbestic point was observed at 230 nm on the CD spectra (*arrow* in Fig. 1*B*) over the concentration range of urea up to 4 M, but not above 4 M, thus showing 2 phases of the structural changes in L-PGDS. The CD spectra in the unfolding state of L-PGDS induced by GdnHCl (17) also showed an isosbestic point over the range up to 2 M, but not above 2 M, giving almost the same propensity of the 2 phases of the unfolding process as those induced by urea. Furthermore, the structural reversibility was confirmed in the CD spectra by the depletion of GdnHCl or urea (data not shown).

Fig. 1*C* shows the equilibrium transition curves of L-PGDS obtained followed by treatment with GdnHCl and urea moni-

[2] M. Emi, D. Irikura, T. Inui, and Y. Urade, unpublished observation.

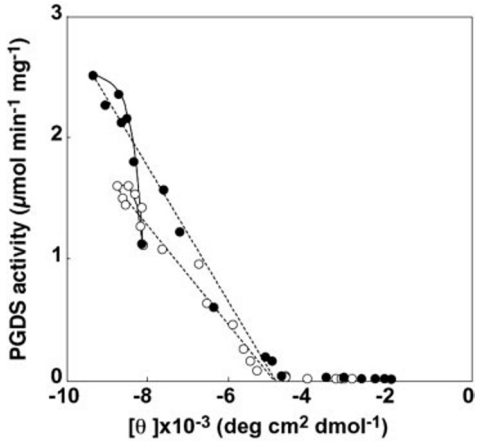

FIG. 2. **Correlation between the PGDS activity and the secondary structure.** The enzyme activities were plotted against the CD ellipticities (θ) at 218 nm in the presence and absence of various concentrations of urea (○) or GdnHCl (●). The correlation factors (r^2) of *dotted lines* with GdnHCl and urea were 0.984 and 0.974, respectively.

tored at the wavelength of 218 nm, which reflects the content of β-sheet structure. The absolute intensity of CD at 218 nm was increased by 0.2–0.8 M GdnHCl and 0.25–1.75 M urea. The absolute intensity reached a maximum at 0.5 M GdnHCl or 1 M urea, and decreased above 0.5 M GdnHCl or 1 M urea in a concentration-dependent manner. Above 1 M GdnHCl or 2 M urea, the transition of L-PGDS was shown to occur in a biphasic manner, having a shoulder around 2 M GdnHCl or 3.5 M urea with respect to the formation of non-native state, as described later.

Two Equilibrium States of L-PGDS in the Unfolding Process—To investigate the correlation between the enzyme activity and the content of β-sheet structure, we plotted the PGDS activities against the ellipticities of the CD spectra of L-PGDS at 218 nm in the presence or absence of GdnHCl or urea (Fig. 2). Nonlinear but ellipticity-dependent activation was observed in the presence of GdnHCl up to 0.5 M or urea up to 1 M (*solid lines*). After showing the maximum activity at the ellipticities of -9.2 and -8.7×10^3 deg cm^2 dmol^{-1} in the presence of 0.5 M GdnHCl and 1 M urea, respectively, the PGDS activity decreased linearly in an ellipticity-dependent manner with a horizontal intercept at approximately -5×10^3 deg cm^2 dmol^{-1} ellipticity in the presence of 2 M GdnHCl and 4 M urea (*dotted lines*). Although the slopes of the linear phase were different between GdnHCl and urea, the enzyme activity was highly dependent on the ellipticity at 218 nm. When the ellipticity was higher than -5.0×10^3 deg cm^2 dmol^{-1}, however, the enzyme activity was below the detection limit. These results, taken together, show that the unfolding of L-PGDS in the presence of denaturants proceeded via two states, *i.e.* an activity-enhanced one and an inactive one.

Table II summarizes the contents of the secondary structure of L-PGDS of the native state in the absence of denaturants and those of the activity-enhanced state in the presence of low concentrations of denaturants calculated from the CD spectra by the method of Chen *et al.* (29). The native L-PGDS was composed of 17% α-helix, 45% β-sheet, and 38% coil. The secondary structure contents of L-PGDS are similar to those of β-lactoglobulin, a member of the lipocalin family, estimated from both CD spectra and x-ray crystallography (30). In the presence of 0.5 M GdnHCl or 1 M urea, the β-sheet content increased from 45 to 51 or 50%, respectively, whereas the coil contents decreased from 38 to 32 or 32%, respectively, without any changes in the α-helical structures. The secondary structure contents of the inactive intermediate in the presence of 2 M GdnHCl or 4 M urea could not be calculated, because of the highly deviated CD spectra.

TABLE II
Contents of secondary structure of L-PGDS calculated from the CD spectra

The contents of secondary structure elements of L-PGDS were estimated by the method of Chen *et al.* (29).

Denaturant	α-Helix	β-Sheet	Coil
		%	
(−)	17	45	38
0.5 M GdnHCl	17	51	32
1 M Urea	18	50	32

Structural Changes in the Activity-enhanced and Inactive States of L-PGDS by Denaturants—We then recorded the ^{15}N-^{1}H heteronuclear multiple quantum coherence spectra of the recombinant mouse L-PGDS in the absence or presence of 0.5 and 2 M GdnHCl or 1 and 4 M urea to detect the signals of amide protons slowly exchanging with the hydrogen bond (Figs. 3, *A-E*). In the ^{15}N-^{1}H heteronuclear multiple quantum coherence spectrum, the positions of several cross-peaks were changed in the activity-enhanced state of L-PGDS formed in the presence of 0.5 M GdnHCl or 1 M urea (Fig. 3, *B* and *C*), whereas their intensities were close to those observed in the spectrum without denaturants. In addition, new cross-peaks were observed in the spectrum of the activity-enhanced state (*arrowheads* in Fig. 3, *B* and *C*), indicating that the hydrogen-bond network in the L-PGDS molecule was reorganized by these denaturants. The number of observed cross-peaks was drastically decreased but still remained in the inactive state in the presence of 2 M GdnHCl (Fig. 3*D*) or 4 M urea (Fig. 3*E*). This phenomenon is interpreted as the loss of hydrogen bonds, and is typical of the denaturation process.

We also determined one-dimensional proton NMR spectra to obtain useful gross information on the conformational status of the two equilibrium states of L-PGDS in the absence and presence of 0.5 to 6 M GdnHCl (Fig. 4). One-dimensional ^{1}H NMR spectra of L-PGDS in the absence or presence of 0.5 M GdnHCl were well dispersed, which reflect the compact folded states of the protein. These features were notably evident in the amide and aromatic (7.5 to 10.0 ppm, Fig. 4*A*) and the aliphatic (0.5 to 3.0 ppm, Fig. 4*B*) regions of the one-dimensional ^{1}H NMR spectrum of the protein. One-dimensional ^{1}H NMR spectra of L-PGDS in the presence of 2 M GdnHCl showed limited chemical shift dispersion in the ^{1}H dimension, which reflects the overall similarity to an unfolded state in the presence of 6 M GdnHCl (Fig. 4, *A* and *B*).

Binding Affinities of L-PGDS for Lipophilic Ligands in the Presence of Denaturants—To investigate the binding affinity of the activity-enhanced state of L-PGDS for small lipophilic ligands, we measured the fluorescence quenching of the intrinsic tryptophan residue of L-PGDS in the presence or absence of 1 M urea or 0.5 M GdnHCl after incubating the enzyme with various concentrations of bilirubin or 13-*cis*-retinal. The recombinant mouse L-PGDS in the absence of these denaturants showed fluorescence quenching in a concentration-dependent manner after addition of bilirubin or 13-*cis*-retinal (Fig. 5*A*). The fluorescence intensity in the presence of an excess amount of bilirubin (above 2 μM) or 13-*cis*-retinal (above 6 μM) decreased below 10 and 30% of the fluorescence intensity in the absence of lipophilic ligands, respectively. In the presence of 1 M urea, bilirubin and 13-*cis*-retinal showed fluorescence quenching curves almost identical to those in the absence of urea (Fig. 5*B*). However, in the presence of 0.5 M GdnHCl (Fig. 5*C*), L-PGDS required higher concentrations of bilirubin to quench the fluorescence by an amount comparable with that in

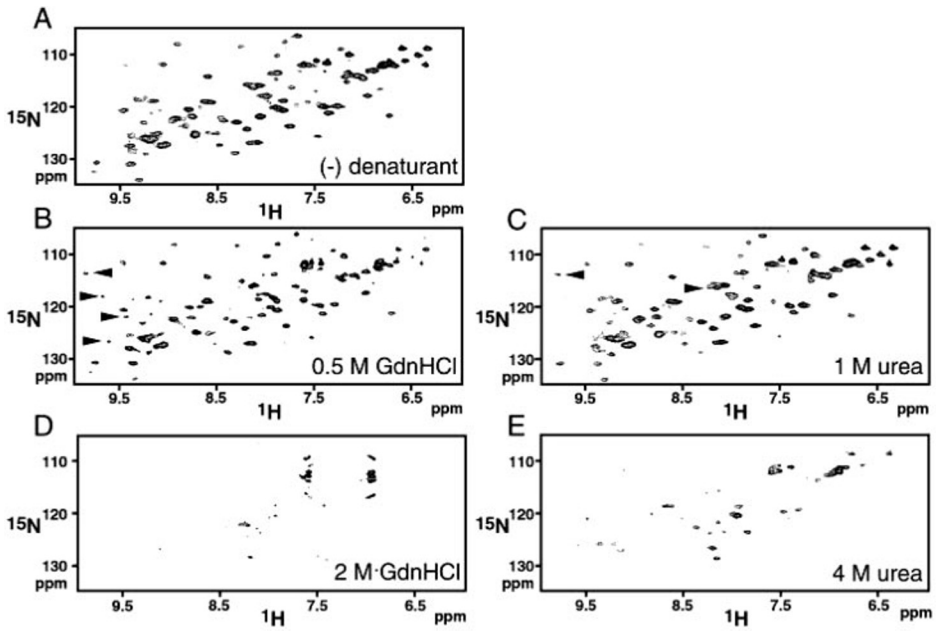

FIG. 3. **^{15}N-^{1}H heteronuclear multiple quantum coherence spectra of L-PGDS in the presence or absence of denaturants.** The spectra were obtained in the absence (*A*) or presence of 0.5 M (*B*) or 2 M (*C*) GdnHCl, and 1 M (*D*) or 4 M (*E*) urea. *Arrowheads* show the new peaks that appeared in the presence of denaturants.

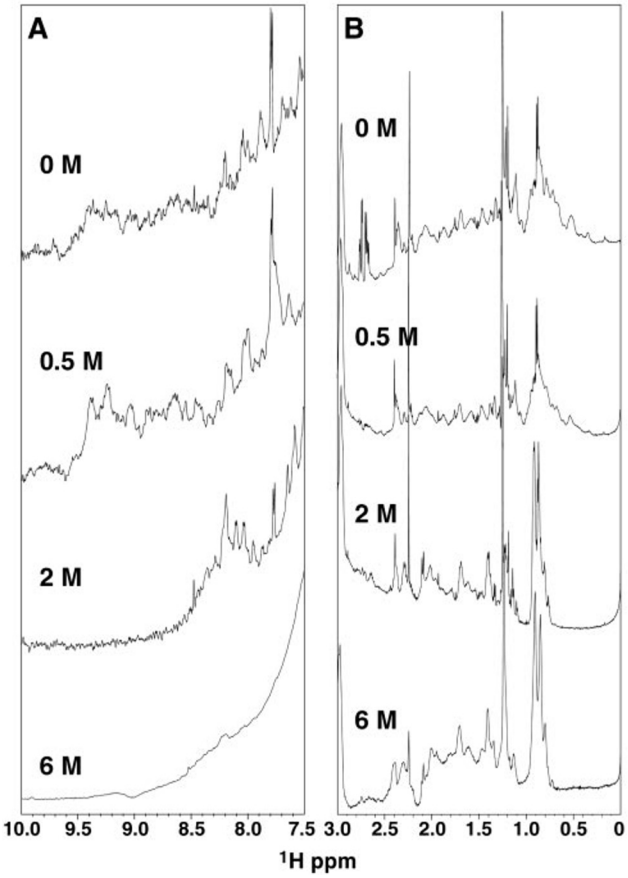

FIG. 4. **Structural changes in the equilibrium states of L-PGDS monitored by NMR spectra.** Amide and aromatic regions (*A*) and aliphatic regions (*B*) of one-dimensional ^{1}H NMR spectra of L-PGDS in the presence or absence of GdnHCl. The spectra were obtained in the absence or presence of 0.5, 2, or 6 M GdnHCl. The concentration of the protein used was 250 μg/ml.

the absence of GdnHCl, whereas the fluorescence quenching curve with 13-*cis*-retinal was almost identical to that in the absence of denaturants.

The calculated K_d values obtained by the method of Levine (26) are also summarized in Table I. In the presence of 1 M urea, the binding affinity of L-PGDS for bilirubin (K_d = 120 nM) was almost identical to that in the absence of denaturant (K_d = 110 nM). However, the affinity in the presence of 0.5 M GdnHCl (K_d = 450 nM) was significantly lower than that in the absence of denaturants. In the case of 13-*cis*-retinal, the K_d values were estimated to be 1.0 μM in the absence of denaturants, 1.1 μM in the presence of 1 M urea, and 1.2 μM in the presence of 0.5 M GdnHCl. The fluorescence quenching was not observed in the inactive state of L-PGDS after denaturation in the presence of 4 M urea or 2 M GdnHCl (data not shown). The binding affinity for bilirubin was lower in the presence of 0.5 M GdnHCl than in the absence of denaturants, but unchanged in the presence of 1 M urea, suggesting that 0.5 M GdnHCl appears to have affected more effectively the conformation of the bilirubin-binding site of L-PGDS than 1 M urea.

Mouse L-PGDS contains two tryptophan residues at positions 43 and 54. To investigate which tryptophan residue contributes to the fluorescence quenching by the small lipophilic ligands, we generated two new mutants of L-PGDS, in which each of these two tryptophan residues was substituted by phenylalanine. Although both mutants of L-PGDS showed fluorescence quenching in a concentration-dependent manner after addition of bilirubin (Fig. 6*A*) and 13-*cis*-retinal (Fig. 6*B*), the W54F mutant showed the more intense tryptophan fluorescence than that of the W43F mutant. The calculated K_d values of W54F mutant and W43F mutant for bilirubin were 109 and 150 nM, respectively, and those for 13-*cis*-retinal were 0.9 and 1.1 μM, respectively. These results indicated that the Trp^{43} residue is located in the more hydrophobic region within a molecule of L-PGDS than the Trp^{54} residue.

Changes in the Binding Mode of L-PGDS for Lipophilic Ligands from Multistate Mode in the Native Form to Simple Two-state Mode in the Activity-enhanced Form—We then compared the binding mode of ligands to the recombinant mouse L-PGDS between the native and the activity-enhanced forms by measuring CD spectra in near-UV and visible ranges. In the absence of denaturants, within the range of the molar ratio of bilirubin/L-PGDS from 0.2 to 0.6, bilirubin bound to L-PGDS

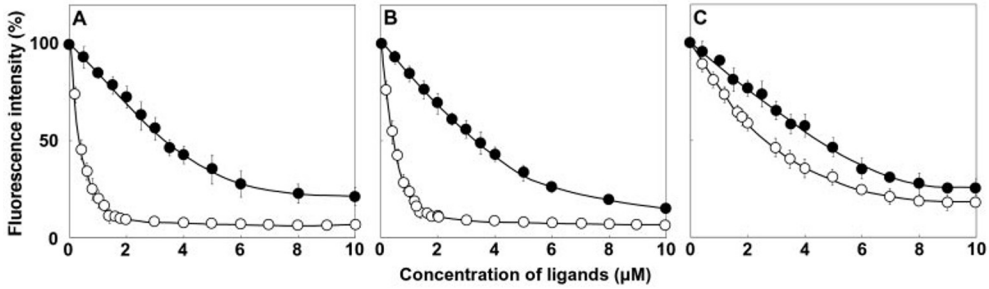

FIG. 5. **Tryptophan fluorescence quenching by lipophilic small ligands.** The relative fluorescence intensities of L-PGDS in the presence of various concentrations of bilirubin (○) or 13-*cis*-retinal (●) were obtained in the absence (*A*) and presence of 1 M urea (*B*) or 0.5 M GdnHCl (*C*). Data are expressed as mean ± S.E. of three independent experiments.

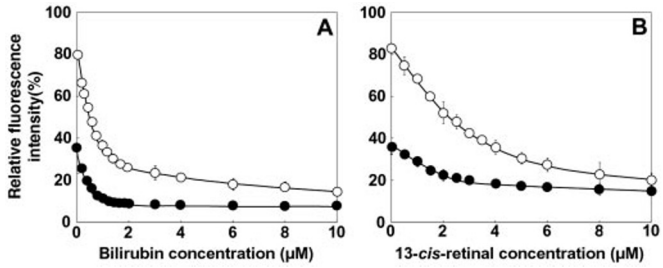

FIG. 6. **Tryptophan fluorescence quenching of the W43F and W54F mutants by lipophilic small ligands.** The relative fluorescence intensities of W43F (●) and W54F (○)-substituted L-PGDS were measured in the presence of bilirubin (*A*) or 13-*cis*-retinal (*B*). Data were expressed as percentages of those with the wild-type enzyme (mean ± S.E., $n = 3$).

showed both negative and positive CD Cotton effects at peaks of 394 and 509 nm, respectively, giving an isosbestic point at 450 nm (*open arrow* in Fig. 7*A*). At the molar ratio of 0.8, new negative and positive peaks appeared at wavelengths of 282 and 461 nm, respectively. Above this molar ratio, the intensity of the positive peak at 509 nm was decreased and that of the other positive peak at 461 nm was increased; and the negative peak at 394 nm was decreased and blue-shifted to 389 nm. The CD spectral changes of bilirubin bound to L-PGDS did not show any isosbestic point above the molar ratio of 0.8. These results suggest that the bilirubin binding mode for the native L-PGDS in the absence of denaturants was not a simple two-state but a multistate one.

On the other hand, in a case of the activity-enhanced form produced in the presence of 1 M urea, the intensities of both negative (284 and 411 nm) and positive (506 nm) CD Cotton effects increased in a molar ratio-dependent manner with an isosbestic point at 460 nm (*open arrow* in Fig. 7*B*). At the molar ratio of 1.6, the intensity of the negative Cotton effect at 411 nm was ~1.7-fold higher than that in the absence of urea (Fig. 7, *A* and *B*). In the presence of 0.5 M GdnHCl, in which the activity-enhanced form also exists, the negative Cotton effects with peaks at 282 and 401 nm and a positive effect with a peak at 515 nm were also found to be increased with a tight isosbestic point at 474 nm (*open arrow* in Fig. 7*C*) similar to that in the presence of 1 M urea. However, the intensity of the negative Cotton effect at 401 nm was almost identical to that in the absence of GdnHCl. These results suggest that the bilirubin binding mode of the activity-enhanced form of L-PGDS conformed to a simple two-state model.

When 13-*cis*-retinal was used as a ligand bound to L-PGDS, negative CD Cotton effects were observed at wavelengths of 260 and 355 nm. In the absence of denaturants, the Cotton effect was increased in a molar ratio-dependent manner up to the molar ratio of 2 without any isosbestic point (Fig. 7*D*). In the presence of 1 M urea, the negative Cotton effects at 260 and 370 nm also increased in a molar ratio-dependent manner without any isosbestic point (Fig. 7*E*). At the molar ratio of 2.0, the intensity of the negative Cotton effect at 370 nm was ~1.3-fold higher than that in the absence of urea. In the presence of 0.5 M GdnHCl, the negative Cotton effects at 260 and 375 nm increased in a molar ratio-dependent manner with a tight isosbestic point at 290 nm (*open arrow* in Fig. 7*F*). These results indicate that in the presence of low concentrations of GdnHCl the binding mode between 13-*cis*-retinal and the activity-enhanced form of L-PGDS was a simple two-state one.

DISCUSSION

Two Equilibrium States of L-PGDS in the Reversible Folding Process—We showed that two states were formed during the process of unfolding of L-PGDS induced by GdnHCl or urea. Based on these findings, we propose the triangle and pole model for L-PGDS (Fig. 8). In this scheme, both *A* and *I* are equilibrium states from the native (*N*) state to the unfolded (*U*) state, which are associated with the changes in the enzyme activity representing both activation and inactivation, respectively. The A state is the activity-enhanced form with a rigid native-like tertiary structure enriched in β-sheet conformation formed maximally in the presence of 0.5 M GdnHCl or 1 M urea, giving the ellipticities of -9.2 or -8.7×10^3 deg cm^2 dmol^{-1}, respectively (Figs. 1 and 2). On the other hand, the I state is the inactive state without the enzyme activity and the ligand-binding activity but possessing the core secondary structure maximally formed in the presence of 2 M GdnHCl or 4 M urea, giving the ellipticity of -4.8×10^3 deg cm^2 dmol^{-1} (Figs. 1–4). The U state is found in the presence of excess amounts of denaturants, exhibits the ellipticity above -4.8×10^3 deg cm^2 dmol^{-1}, and is nonrelevant to enzyme activity and ligand binding. In the present study, we proposed that both A and I equilibrium states were located on a sequential pathway from the N to U state. However, it is still unclear whether the A state is an on-pathway intermediate from the N to I state. The N and A state each may convert directly to the I state. As a minor possibility, the A state must convert back to the N state before converting to the I state.

To the best of our knowledge, L-PGDS is the first example of multiphasic equilibrium states with the A and I states in the unfolding process of a β-barrel protein; whereas a number of other β-barrel proteins, such as β-lactoglobulin, cellular retinoic acid-binding protein, and human acidic fibroblast growth factor, have been shown to fold in a two-state transition (31). For example, human acidic fibroblast growth factor forms a stable equilibrium intermediate accumulated maximally at 0.96 M GdnHCl resembling a molten globule state as examined by CD, fluorescence, and NMR (32). The molten globule is found in various proteins, such as β-lactoglobulin, cytochrome *c*, and α-lactalbumin, and is proposed to exist in the partially folded equilibrium state of the protein with pronounced secondary structure but no rigid tertiary structure (33–35). Thus, we consider the I state of L-PGDS to be a molten globule-like state

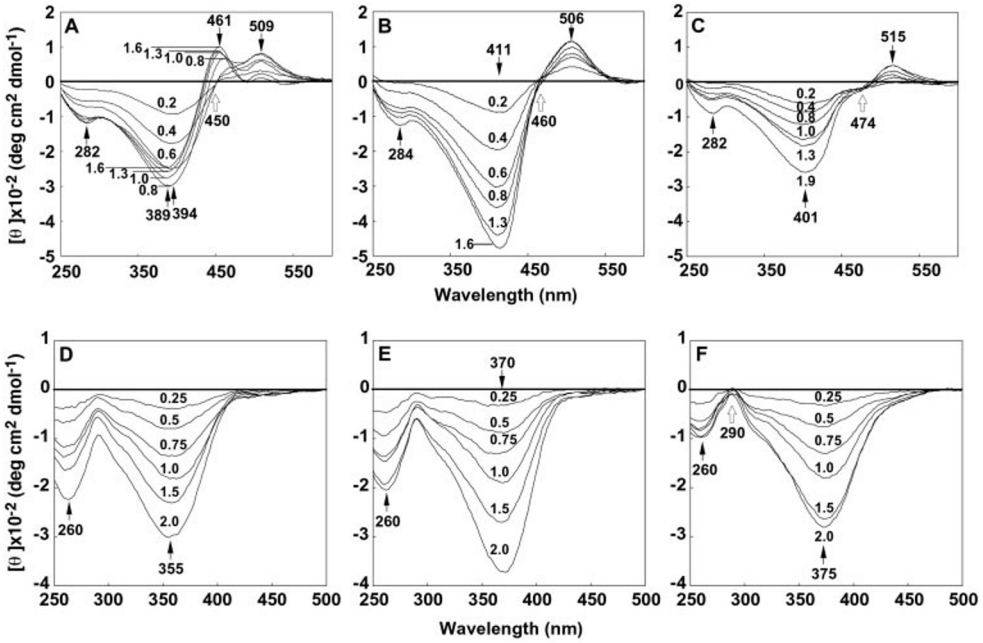

FIG. 7. **CD spectra in near-UV and visible range of lipophilic small ligands bound to L-PGDS.** CD spectra of bilirubin bound to L-PGDS (10 μM) without denaturant (*A*), in the presence of 1 M urea (*B*), and in the presence of 0.5 M GdnHCl (*C*) were recorded. CD spectra of 13-*cis*-retinal bound to L-PGDS (20 μM) without denaturant (*D*), in the presence of 1 M urea (*E*), and in the presence of 0.5 M GdnHCl (*F*) were also prepared. *Numbers* indicate the molar ratios of ligand to L-PGDS. *Closed arrows* indicate the peaks of the Cotton effects; and *open arrows*, the isosbestic points.

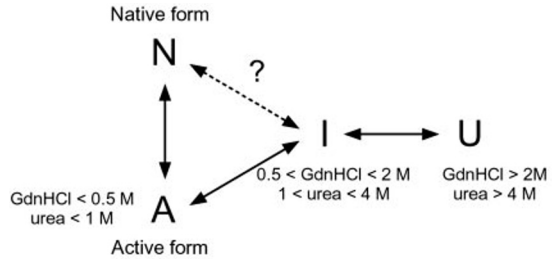

FIG. 8. **A model of the reversible folding and unfolding process of L-PGDS.** *N*, *A*, *I*, and *U* represent the native, activity-enhanced, inactive, and unfolded states of L-PGDS, respectively.

because it contained pronounced secondary structure without rigid tertiary structure and apparent L-PGDS function. The molten globule has been proposed to be involved in a number of physiological processes, such as protein recognition by chaperones, release of protein ligands, and protein translocation across biomembranes (36, 37). L-PGDS is secreted into various body fluids such as cerebrospinal fluid, interphotoreceptor matrix, seminal plasma, and plasma, after cleavage of its N-terminal signal peptide to bind and transfer various small lipophilic ligands such as retinoids, bile pigments, and thyroid hormones (1, 2). The L-PGDS concentration is increased in the cerebrospinal fluid of patients after subarachnoid hemorrhage (38, 39), and the enzyme is produced by leptomeningeal cells of adult rats, secreted in the cerebrospinal fluid, and taken up by macrophages to be accumulated in their lysosome, as concluded from examination by confocal immunofluorescence microscopy (40). Therefore, the I state of L-PGDS may also be involved in protein translocation across biomembranes of the leptomeningeal cells and in the release of the bound ligands within the lysosome.

Correlation between Enzyme Activity and Conformational Changes in the Presence of Denaturants—We showed that the L-PGDS activity was increased 2.5- and 1.6-fold in the presence of 0.5 M GdnHCl or 1 M urea, respectively (Fig. 1), mainly because of an increase in k_{cat} of the A state (Table I). Moreover, an increase in the binding affinity of the A state for the substrate, as shown by the decreased K_m value, may also contribute to the enhancement of L-PGDS activity by urea. Activation of an enzyme by denaturants has been reported in the case of only a few enzymes, such as adenylate kinase and dihydrofolate reductase. The adenylate kinase activity was enhanced 1.6-fold in the presence of 1 M urea or 0.25 M GdnHCl as compared with that in the absence of denaturants (19). Dihydrofolate reductase was also activated about 2-fold in the presence of 0.5 M GdnHCl and about 5-fold in the presence of 4 M urea (20, 21), the latter of which was associated with an increase in the k_{cat} value (20). However, the overall structures of adenylate kinase and dihydrofolate reductase were not significantly altered within the indicated concentration ranges of denaturants as monitored by CD and UV spectra (19–21). The change in the enzyme activity of adenylate kinase in the presence of the low concentration of denaturants coincided with that in the binding rate of 8-anilino-1-naphthalenesulfonic acid (19). The activation of dihydrofolate reductase was accompanied by an increase in the rate of digestion by trypsin (20, 21). Therefore, the activation of these enzymes was proposed to be caused by an increase in the conformational flexibility locally at the active site. On the other hand, L-PGDS clearly changed its secondary and tertiary structures in the presence of low concentrations of denaturants to be highly correlated with the β-sheet content, as monitored by the CD spectra at 218 nm (Fig. 2). The reorganization of the hydrogen-bond network was also associated with the activation of L-PGDS in the presence of urea or GdnHCl, as revealed by the NMR spectra (Figs. 3 and 4). As judged from our recent x-ray crystallographic analyses, L-PGDS contains a well ordered hydrophobic coil structure near the β-sheet.[3] In the presence of low concentrations of the denaturants, the hydrophobic interaction is weakened to transform a part of the coil structure to the β-sheet, which is stabilized by a newly formed hydrogen bonding network. Such a rearrangement of the L-PGDS structure might occur *in vivo* by

[3] D. Irikura and Y. Urade, unpublished results.

binding of metal ions or other substances.

Correlation between Ligand-binding Activity and Conformational Changes in the Presence of Denaturants—The fluorescence quenching study on the intrinsic tryptophan residue of L-PGDS (Fig. 5 and Table I) revealed that the K_d value of L-PGDS for bilirubin was almost unchanged from the N state (110 nM) to the A state in the presence of 1 M urea (120 nM) but remarkably increased in the A state in the presence of 0.5 M GdnHCl (450 nM). On the other hand, the K_d value for 13-*cis*-retinal was almost identical either in the absence or presence of denaturants (Fig. 5 and Table I), suggesting that the N and A states of L-PGDS possess distinct binding modes against these 2 ligands within the hydrophobic pocket. The CD spectra of bilirubin and 13-*cis*-retinal bound to the A form of L-PGDS showed well defined isosbestic points (Fig. 6), demonstrating that the binding mode of ligands to L-PGDS changed from a multistate binding mode in the N state to a simple two-state mode in the A state. The simple two-state binding mode at the A state may also reflect the effective interaction between PGH_2 and L-PGDS, whose activity was enhanced by denaturants. The K_m value of L-PGDS for PGH_2 was also almost unchanged from the N state (2.8 mM) to the A state in the presence of 1 M urea (2.3 mM) but clearly increased in the A state in the presence of 0.5 M GdnHCl (8.3 mM, Table I). The structural change near the active thiol of Cys^{65} at the entrance of the hydrophobic pocket in the A state of L-PGDS is considered to be induced by 0.5 M GdnHCl more significantly than by 1 M urea, as was shown by the slight difference in the curves of the activity-enhanced L-PGDS obtained with the 2 kinds of denaturants (Figs. 1 and 2).

Most recently, the x-ray crystallographic analysis of L-PGDS revealed that the Trp^{43} residue is located at the bottom of the hydrophobic pocket, whereas the Trp^{54} residue locates on the loop position and faces the outside of the hydrophobic pocket.[4] Therefore, it is likely that the Trp^{43} residue is considered to be responsible for the major portion of fluorescence quenching by the binding of ligands. In this study, we showed that both tryptophan residues contributed to the fluorescence quenching of L-PGDS by the ligand binding, although the Trp^{43} residue showed the more intense fluorescence than that of the Trp^{54} residue. The Trp^{54} residue might move to the lipophilic ligand entered at the hydrophobic pocket of L-PGDS and weakly contribute to the fluorescence quenching.

L-PGDS binds various hydrophobic ligands, such as retinoids, bilirubin, biliverdin, and thyroid hormones, with high affinities (K_d = 30 ~ 200 nM, Refs. 9, 10, and 17). This broad selectivity may reflect a general transport role as a lipocalin for the clearance of unwanted endogenous or exogenous lipophilic molecules. β-Lactoglobulin, a member of the lipocalin family, also exhibits a broad selectivity of ligand binding (41), but it does not bind bilirubin or biliverdin (data not shown). In previous unfolding studies on β-lactoglobulin by CD analysis (30, 42), the ellipticities at 219 and 222 nm were shown to be increased in the presence of GdnHCl up to 2.5 M, as compared with the value in the absence of GdnHCl. Although the increase in the secondary structure of β-lactoglobulin was not examined in those studies, such results indicate that the β-sheet content in β-lactoglobulin was increased by low concentrations of GdnHCl and suggest that the activity enhanced-like equilibrium state was also formed during the unfolding of β-lactoglobulin. On the other hand, neither retinoic acid- nor retinol-binding proteins showed any increase in ellipticity at 218 nm on CD spectra in the presence of urea up to 9 M (43). The increase in the secondary structure by low concentrations of denaturants may not be a feature of all molecules of the lipocalin family. Therefore, L-PGDS is a unique protein that possesses two equilibrium states: the activity-enhanced and the inactive states, in a reversible folding process and shows a high correlation between structural changes in itself and its dual functions of enzyme activity and ligand binding.

[4] D. Irikura and Y. Urade, unpublished results.

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REFERENCES

1. Urade, Y., and Hayaishi, O. (2000) *Biochim. Biophys. Acta* **1482,** 259–271
2. Urade, Y., and Eguchi, N. (2002) *Prostaglandins Other Lipid Mediat.* **68–69,** 375–382
3. Urade, Y., Fujimoto, N., and Hayaishi, O. (1985) *J. Biol. Chem.* **260,** 12410–12415
4. Matsuoka, T., Hirata, M., Tanaka, H., Takahashi, Y., Murata, T., Kabashima, K., Sugimoto, Y., Kobayashi, T., Ushikubi, F., Aze, Y., Eguchi, N., Urade, Y., Yoshida, N., Kimura, K., Mizoguchi, A., Honda, Y., Nagai, H., and Narumiya, S. (2000) *Science* **287,** 2013–2017
5. Toh, H., Kubodera, H., Nakajima, N., Sekiya, T., Eguchi, N., Tanaka, T., Urade, Y., and Hayaishi, O. (1996) *Protein Eng.* **9,** 1067–1082
6. Flower, D. R., North, A. C., and Sansom, C. E. (2000) *Biochim. Biophys. Acta* **1482,** 9–24
7. Flower, D. R. (1996) *Biochem. J.* **318,** 1–14
8. Pervaiz, S., and Brew, K. (1987) *FASEB J.* **1,** 209–214
9. Tanaka, T., Urade, Y., Kimura, H., Eguchi, N., Nishikawa, A., and Hayaishi, O. (1997) *J. Biol. Chem.* **272,** 15789–15795
10. Beuckmann, C. T., Aoyagi, M., Okazaki, I., Hiroike, T., Toh, H., Hayaishi, O., and Urade, Y. (1999) *Biochemistry* **38,** 8006–8013
11. Monaco, H. L., Zanotti, G., Spadon, P., Bolognesi, M., Sawyer, L., and Eliopoulos, E. E. (1987) *J. Mol. Biol.* **197,** 695–706
12. Cowan, S. W., Newcomer, N. E., and Jones, T. A. (1990) *Proteins* **8,** 44–61
13. Böcskel, Z., Groom, C. R., Flower, D. R., Wright, C. E., Philips, S. E. V., Cavaggioni, A., Findlay, J. B. C., and North, A. C. T. (1992) *Nature* **360,** 186–188
14. Newcomer, M. E., Pappas, R. S., and Ong, D. E. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9223–9227
15. Weichsel, A., Andersen, J. F., Champagne, D. E., Walker, F. A., and Montfort, W. R. (1998) *Nat. Struct. Biol.* **5,** 304–309
16. Urade, Y., Tanaka, T., Eguchi, N., Kikuchi, M., Kimura, H., Toh, H., and Hayaishi, O. (1995) *J. Biol. Chem.* **270,** 1422–1428
17. Inui, T., Ohkubo, T., Urade, Y., and Hayaishi, O. (1999) *Biochem. Biophys. Res. Commun.* **266,** 641–646
18. Tsou, C.-L. (1993) *Science* **262,** 380–381
19. Zhang, H.-J., Sheng, X.-R., Pan, X.-M., and Zhou, J.-M. (1997) *Biochem. Biophys. Res. Commun.* **238,** 382–386
20. Fan, Y. X., Ju, M., Zhou, J. M., and Tsou, C. L. (1995) *Biochim. Biophys. Acta* **1252,** 151–157
21. Fan, Y.-X., Ju, M., Zhou, J.-M., and Tsou, C.-I. (1996) *Biochem. J.* **315,** 97–102
22. Hoffmann, A., Bächner, D., Betar, N., Lauber, J., and Gross, G. (1996) *Dev. Dyn.* **207,** 332–343
23. Oda, H., Eguchi, N., Urade, Y., and Hayaishi, O. (1996) *Proc. Jpn Acad.* **72,** 108–111
24. McDonagh, A. F., and Assisi, F. (1971) *FEBS Lett.* **18,** 315–317
25. Robert, S. H., Liu, S. H., and Asato, A. E. (1982) *Methods Enzymol.* **88,** 506–516
26. Levine, R. L. (1977) *Clin. Chem.* **23,** 2292–2301
27. Bax, A., Griffey, R. H., and Hawkins, B. L. (1983) *J. Magn. Reson.* **55,** 301–315
28. Molday, R. S., Englander, S. W., and Kallen, R. G. (1972) *Biochemistry* **11,** 150–159
29. Chen, Y. H., Yang, J. T., and Chau, K. H. (1974) *Biochemistry* **13,** 3350–3359
30. Kuwajima, K., Yamaya, H., and Sugai, S. (1996) *J. Mol. Biol.* **264,** 806–822
31. Capaldi, A., and Radford, S. (1998) *Curr. Opin. Struct. Biol.* **8,** 86–92
32. Samuel, D., Kumar, T. K., Srimathi, T., Hsieh, H., and Yu, C. (2000) *J. Biol. Chem.* **275,** 34968–34975
33. Ptitsyn, O. B. (1992) in *Protein Folding* (Creighton, T. E., ed) pp. 243–300, W. H. Freeman Co., New York
34. Jeng, M. F., and Englander, S. W. (1991) *J. Mol. Biol.* **221,** 1045–1061
35. Hughson, F. M., Wright, P. E., and Baldwin, R. L. (1990) *Science* **249,** 1544–1548
36. Buchanan, S. (1999) *Curr. Opin. Struct. Biol.* **9,** 455–461
37. Fersht, A., and Daggett, V. (2002) *Cell* **108,** 573–582
38. Mase, M., Yamada, K., Iwata, A., Matsumoto, T., Seiki, K., Oda, H., and Urade, Y. (1999) *Neurosci. Lett.* **270,** 188–190
39. Inui, T., Mase, M., Emi, M., Nakau, H., Seiki, K., Oda, H., Yamada, K., and Urade, Y. (2003) in *Oxygen and Life-Oxygenases, Oxydases and Lipid Mediators* (Ishimura, Y., ed) Vol. 1233C, pp. 447–451, Elsevier, Amsterdam
40. Beuckmann, C., Lazarus, M., Gerashchenko, D., Mizoguchi, A., Nomura, S., Mohri, I., Uesugi, A., Kaneko, T., Mizuno, N., Hayaishi, O., and Urade, Y. (2000) *J. Comp. Neurol.* **428,** 62–78
41. Akerstrom, B., Flower, D. R., and Salier, J. P. (2000) *Biochim. Biophys. Acta* **1482,** 1–8
42. Hamada, D., and Goto, Y. (1997) *J. Mol. Biol.* **269,** 479–487
43. Burns, L. L., Dalessio, P. M., and Ropson, I. J. (1998) *Proteins* **33,** 107–118