Binding of Biliverdin, Bilirubin, and Thyroid Hormones to Lipocalin-Type Prostaglandin D Synthase†

Carsten T. Beuckmann,‡ Masaaki Aoyagi,§ Issay Okazaki,|| Takaaki Hiroike,⊥ Hiroyuki Toh,⊥ Osamu Hayaishi,‡ and Yoshihiro Urade*,‡,#

Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565-0874, Japan, Nissei Sangyo Company Ltd., ULALA 3 9-3, Yamato-cho, Tsuchiura, Ibaraki 300-0036, Japan, Biacore K.K., 2-9-5 Shiba Koen, Minato-ku, Tokyo 105-0011, Japan, Biomolecular Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565-0874, Japan, and CREST, Japan Science Technology Corporation, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565-0874, Japan

Received February 4, 1999

ABSTRACT: Lipocalin-type prostaglandin D synthase is a major protein of the cerebrospinal fluid and was originally known as β-trace. We investigated the binding ability of prostaglandin D synthase toward bile pigments, thyroid hormones, steroid hormones, and fatty acids in this present study. We found that the recombinant enzyme binds bile pigments and thyroid hormones, resulting in quenching of the intrinsic tryptophan fluorescence, the appearance of induced circular dichroism of the lipophilic ligands, and a red shift of the absorption spectra of bilirubin and biliverdin. The binding of prostaglandin D synthase to lipophilic ligands was also demonstrated by the resonant mirror technique and surface plasmon resonance detection. The dissociation constants were calculated to be 33 nM, 37 nM, 660 nM, 820 nM, and 2.08 μM for biliverdin, bilirubin, L-thyroxine, 3,3′,5′-triiodo-L-thyronine, and 3,3′,5-triiodo-L-thyronine, respectively. Biliverdin and bilirubin underwent a shift in their absorption peaks from 375 to 380 nm and from 439 to 446 nm, respectively, after binding to prostaglandin D synthase. Bilirubin bound to the enzyme showed a bisignate CD spectrum with a (−) Cotton effect at 422 nm and a (+) Cotton effect at 472 nm, indicating a right-handed chirality. The ligands also inhibited prostaglandin D synthase activity noncompetitively in a concentration-dependent manner, with IC_{50} values between 3.9 and 10.9 μM. Epididymal retinoic acid-binding protein and β-lactoglobulin, two other lipocalin proteins that bind retinoids such as prostaglandin D synthase, did not show any significant interaction with bile pigments or thyroid hormones. These results show that prostaglandin D synthase binds small lipophilic ligands with a specificity distinct from that of other lipocalins.

Lipocalin-type prostaglandin (PG)[1] D synthase [PGDS; (5*Z*,13*E*)-(15*S*)-9α,11α-epidioxy-15-hydroxyprosta-5,13-dienoate D-isomerase, EC 5.3.99.2] (*1*−*3*) is mainly localized in the mammalian central nervous system (CNS) (*4*−*9*), male genital organs (*4, 7, 10, 11*), and human heart (*12*) and is involved in the biosynthesis of PGD_2 in these tissues. Sequence analyses and database comparisons revealed that PGDS is the only member that displays enzymatic activity among members of the lipocalin superfamily (*13*−*15*), a group of small secretory proteins that bind and transport a large variety of lipophilic biomolecules (*16, 17*).

PGDS has recently been identified to be β-trace (*18*−*21*), which is the most abundant protein in human cerebrospinal fluid (CSF) produced in the CNS (*22*−*24*). Since the substrate of PGDS, PGH_2, is supplied by the action of a microsomal enzyme, cyclooxygenase, and is rapidly degraded in aqueous solution (*25*), it is unlikely that PGDS in the CSF contributes to production of PGD_2. As an alternative function of extracellular PGDS, we proposed previously that PGDS may function as an extracellular retinoid transporter rather than as a PGD_2-producing enzyme (*26*), because the recombinant PGDS binds *all-trans*- or 9-*cis*-retinoic acid and *all-trans*- or 13-*cis*-retinal with affinities (K_d = 70−80 nM) comparable to those of other lipocalins acting as an extracellular retinoid transporter, such as β-lactoglobulin (LG) and plasma retinol-binding protein. However, when we analyzed the endogenous ligand bound to β-trace in human CSF by nuclear magnetic resonance, absorption, and circular dichroism (CD) spectroscopy, we could not detect retinoids

† This work was supported in part by an International Fellowship Grant from Takeda Science Foundation and a Science and Technology Agency (STA) Fellowship (no. 296201 to C.T.B.), a European Union Science and Technology Fellowship (no. ERBIC17CT970050 to C.T.B.), the Ministry of Health and Welfare of Japan (100107 to O.H.), grants-in-aid from the Scientific Research Program of the Ministry of Education, Science, and Culture of Japan (07558108 and 07457033 to Y.U.), and grants from the Sankyou Foundation of Life Science, Japan Foundation for Applied Enzymology, and The Cell Science Research Foundation (to Y.U.).

* To whom correspondence should be addressed: tel, 81-6-6872-4851; fax, 81-6-6872-2841; e-mail, uradey@obi.or.jp.

‡ Osaka Bioscience Institute.

§ Nissei Sangyo Co. Ltd.

|| Biacore K. K.

⊥ Biomolecular Engineering Research Institute.

Japan Science Technology Corp.

[1] Abbreviations: CD, circular dichroism; CNS, central nervous system; CSF, cerebrospinal fluid; DMSO, dimethyl sulfoxide; LG, β-lactoglobulin; PBS, phosphate-buffered saline; PG, prostaglandin; PGDS, prostaglandin D synthase; RABP, epididymal retinoic acid binding protein; reverse T3, 3,3′,5′-triiodo-L-thyronine; T2, 3,5-diiodo-L-thyronine; T3, 3,3′,5-triiodo-L-thyronine; T4, L-thyroxine.

10.1021/bi990261p CCC: $18.00
Published on Web 06/03/1999

but found that bile pigments were bound to β-trace in CSF of several patients after subarachnoidal hemorrhage (C. T. Beuckmann and Y. Urade, unpublished results). Instead of using β-trace purified from human CSF, which is limited in amounts, we employed recombinant enzymes in this study to characterize the binding between lipocalin-type PGDS and bile pigments and several other candidates of its hydrophobic ligands, including thyroid and steroid hormones, iodinated amino acids, and fatty acids.

By fluorescence quenching measurement, CD spectroscopy, absorption spectroscopy, resonant mirror detection, surface plasmon resonance detection, inhibition assays of the enzyme activity, and direct binding assays, we show here that PGDS binds biliverdin and bilirubin with affinities (K_d = 33 and 37 nM, respectively) higher than those for retinoids. We also found that thyroid hormones were bound to PGDS with affinities in a micromolar order. These results suggest that PGDS in the CSF may act as a scavenger for harmful hydrophobic compounds produced in the CNS under pathological conditions and function as a rescue transporter for thyroid hormones in the CNS.

EXPERIMENTAL PROCEDURES

Materials. Biliverdin, bilirubin, L-thyroxine (T4), 3,3′,5-triiodo-L-thyronine (T3), 3,3′,5′-triiodo-L-thyronine (reverse T3), 3,5-diiodo-L-thyronine (T2), 3,5-diiodo-L-tyrosine, 3-iodo-L-tyrosine, *cis*-4,7,10,13,16,19-docosahexaenoic acid, progesterone, 5-pregnen-3β-ol-20-one (pregnenolone), testosterone, 17β-estradiol, *all-trans*-retinoic acid, and *all-trans*-retinol were purchased from Sigma. Bis(2-methoxyethyl) ether was obtained from Tokyo Kasei (Tokyo, Japan) and dimethyl sulfoxide (DMSO) from WAKO (Tokyo, Japan). [1-^{14}C]-PGH_2 was prepared from [1-^{14}C]arachidonic acid (2.20 GBq/mmol; Du Pont-New England Nuclear, Boston, MA) as described (*1*). Cow milk LG was obtained from Sigma. Rat recombinant epididymal retinoic acid-binding protein (RABP) was kindly provided by Dr. T. Tanaka, Biomolecular Engineering Research Institute, Osaka, Japan. All other chemicals were purchased from Sigma unless otherwise indicated.

Expression and Purification of Recombinant Rat Lipocalin-Type PGDS. All amino acid positions referred to in this study were counted starting from the first amino acid, Met^1, of the whole sequence. The recombinantly expressed PGDS lacked the 22 amino acid long N-terminal signal peptide, so that the first three amino acids of the recombinant PGDS were Gly and Ser (from the introduced thrombin cutting site) and Pro^{23}. Due to a very low yield of correctly folded recombinant rat PGDS with three cysteine residues, the Cys-Ala-substituted PGDS were used in this study (*27*). In the Ala^{65} mutant, the Cys^{65} as the active site was replaced so that it lacked the PGDS activity but possessed a cystine bridge between Cys^{89} and Cys^{186}. The Ala^{89,186} mutant maintained the full enzyme activity but lacked the cystine bridge. The Ala^{89,186} and Ala^{65} enzymes were expressed as glutathione transferase fusion proteins by a bacterial expression system. Briefly, the mutated cDNAs were cloned into the *Bam*HI−*Eco*RI site of the pGEX-2T plasmid (Amersham Pharmacia Biotech, Tokyo, Japan) and expressed in *Escherichia coli* DH5α. The fusion proteins were bound to glutathione−Sepharose (Amersham Pharmacia Biotech) and incubated with thrombin to release the mutated PGDS. The recombinant PGDS was further purified by Mono-S column chromatography to apparent homogeneity as judged by silver staining of 100 ng of protein after SDS−PAGE.

Stock Solutions of Lipophilic Ligands. Biliverdin and bilirubin were dissolved in DMSO to give 2 mM stock solutions. Other ligands were dissolved in bis(2-methoxyethyl) ether/ethanol (1:1) to give stock solutions of 4 mM or in DMSO to give stock solutions of 2 mM. The concentrations were determined spectroscopically with ϵ_{377} in methanol for biliverdin = 51 500 $M^{-1}\cdot cm^{-1}$ (*28*), ϵ_{453} in chloroform for bilirubin = 61 700 $M^{-1}\cdot cm^{-1}$ (*29*), ϵ_{325} at pH 11 for T4 = 6 180 $M^{-1}\cdot cm^{-1}$, ϵ_{320} at pH 10 for T3 and reverse T3 = 4660 $M^{-1}\cdot cm^{-1}$ (*30, 31*), ϵ_{336} in ethanol for *all-trans*-retinoic acid = 45 000 $M^{-1}\cdot cm^{-1}$, and ϵ_{325} in ethanol for *all-trans*-retinol = 46 000 $M^{-1}\cdot cm^{-1}$ (*32*). Stock solutions were stored light-protected at −20 °C.

Fluorescence Quenching Assays. Bile pigments, thyroid hormones, T2, 3,5-diiodo-L-tyrosine, 3-iodo-L-tyrosine, steroid hormones, docosahexaenoic acid, *all-trans*-retinoic acid, and *all-trans*-retinol (10 μL) were added to rat recombinant Ala^{65}- or Ala^{89,186}-PGDS, LG, or RABP in 990 μL of 5 mM Tris-HCl, pH 8.0, to give a final concentration of 1.5 μM. After incubation at 20 °C for 60 min, the intrinsic tryptophan fluorescence was recorded in a RF-5000 spectrofluorophotometer (Shimadzu, Kyoto, Japan) with an excitation wavelength at 282 nm. Optimal emission wavelengths were determined to be 338 nm for PGDS, 326 nm for RABP, and 334 nm for LG. The quenching of tryptophan fluorescence due to nonspecific interactions with ligands was corrected with 1.5 μM *N*-acetyl-L-tryptophanamide. The molar ratios and K_d for binding between ligands and PGDS were calculated by the methods of Levine (*33*) or Cogan et al. (*34*).

Solid-Phase Assay for Binding between PGDS and Lipophilic Ligands. The binding of lipophilic ligands to immobilized PGDS was monitored with an IAsys plus device (Affinity Sensors, Cambridge, U.K.) as described (*35*), with some modifications. The Ala^{65}-PGDS [30 μL of 1 mg/mL in phosphate-buffered saline (PBS): 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3 mM KCl, 140 mM NaCl (pH 7.2); Takara, Tokyo, Japan] was immobilized on the surface of an aminosilane-coated sensor activated with 1.6% (v/v) glutardialdehyde, by incubation with the protein solution for 20 min. Nonspecific binding sites were masked with LG (30 μL of 1.5 mg/mL in PBS) by incubation for 20 min, because LG does not bind the bile pigments or the thyroid hormones, as found by fluorescence quenching experiments. The sensor was then washed with 10% (v/v) ethanol in PBS to remove any nonspecifically bound molecules. A reference cuvette was treated identically without immobilization of PGDS. Amounts of proteins immobilized on the sensor surface were determined to be 1.0−1.2 ng/mm^2, resulting in a 600−720 arc second response. For the binding assay, biliverdin (2.5−17.5 μM), bilirubin (5−20 μM), or thyroid hormones (2.5−20 μM) were dissolved in PBS containing 1% (v/v) DMSO. After application of the ligand solution to the immobilized PGDS, the mass increase on the sensor surface due to ligand binding was monitored by measuring the change in the refractive index at the sensor surface with the resonance

mirror detector. The cuvettes were regenerated after each application by washing with 10% (v/v) ethanol in PBS. Data were analyzed by pseudo-first-order approximation (*36*) using FASTfit software. Each measurement was repeated at least three times.

The binding of Ala^{65}-PGDS to the immobilized thyroid hormones was measured with a BIAcore 2000 device (Biacore, Osaka, Japan). The thyroid hormones were covalently coupled via primary amino groups to a carboxymethylated dextran matrix, CM5, as reported previously (*37*, *38*). The matrix was activated with 200 mM *N*-ethyl-*N*′-(3-(dimethylamino)propyl)carbodiimide hydrochloride and 50 mM *N*-hydroxysuccinimide and incubated with various amounts of thyroid hormones followed with 1 M ethanolamine for deactivation of the unused reactive groups. The sensor cells were then washed with 10 mM NaOH. The density of immobilized hormones was 170 pg/mm^2 for T4 and 80 pg/mm^2 for T3 and reverse T3. PGDS (up to 50 μM in PBS) was applied to the immobilized thyroid hormones, and the changes in mass on the sensor surface were monitored by surface plasmon resonance detection. The nonspecific binding was determined by the affinity-in-solution method (*38*) with PGDS preincubated with 10−160-fold excess amounts of thyroid hormone (20 μM). The specific binding responses were calculated by subtracting the nonspecific response from the total response with PGDS without preincubation with thyroid hormones. All binding responses were measured at equilibrium. Data were analyzed by using BIA evaluation 3.0 software. Each measurement was performed at least three times.

UV/Visible Spectra. Spectra of biliverdin and bilirubin (15 μM) in the absence or presence of Ala^{65}-PGDS (15 μM) were recorded five times in a V-560 UV/vis spectrometer (JASCO, Tokyo, Japan) at 25 °C with a bandwidth of 1 nm and a resolution of 1 nm at a scan speed of 100 nm/min. Ligand spectra in the presence of PGDS were corrected for the absorption spectrum of the protein itself.

CD Spectra. Ala^{65}-PGDS was incubated at 10 °C with T4, T3, or reverse T3 in 1 mL of PBS containing 1% (v/v) bis-(2-methoxyethyl) ether/ethanol (1:1) and with bilirubin or biliverdin in PBS containing 1% (v/v) DMSO. CD spectra were recorded in a J-600 spectropolarimeter (JASCO, Tokyo, Japan) at 10 °C. The spectra were accumulated 10 times with a bandwidth of 1 nm and a resolution of 1 nm at a scan speed of 200 nm/min.

Enzyme Assays. PGDS activity was measured with the Ala^{89,186} enzyme (610 nM) by incubation with 40 μM [1-^{14}C]-PGH_2 in 50 μL of 100 mM Tris-HCl, pH 8.0, in the presence of 1 mM dithiothreitol. Bile pigments, thyroid hormones, T2, 3,5-diiodo-L-tyrosine, 3-iodo-L-tyrosine, testosterone, pregnenolone, progesterone, estradiol, or docosahexaenoic acid were added to the reaction mixture to give concentrations up to 20 μM. The reaction was performed for 1 min at 25 °C (*1*, *9*). After thin-layer chromatographic separation of the products from the substrate, the conversion rate was calculated to determine the enzyme activity, as reported previously (*1*). Nonenzymatic degradation of the substrate was determined by omitting PGDS from the reaction mixture. For the competitive inhibition assay, the enzyme (610 nM) was incubated with various concentrations (10−35 μM) of [1-^{14}C]PGH_2 for 30 s at 25 °C in the absence or presence of 2 and 5 μM T4. Protein concentrations were determined by the method of Lowry et al. (*39*).

Fatty Acid Binding Assays. Assays for binding between Ala^{65}-PGDS and arachidonic acid, linoleic acid, or docosahexaenoic acid were carried out as described by Descalzi Cancedda et al. (*40*). Ala^{65}-PGDS was incubated with radiolabeled fatty acids at 4 °C. Free ligand was then trapped by hydroxyalkoxypropyldextran at 4 °C, followed by centrifugation to separate dextran from the protein, and the radioactivity of the supernatant was then counted to determine the amount of fatty acid bound to the protein.

Calculation of a Binding Model between PGDS and Bilirubin. A model structure of PGDS complexed with bilirubin was constructed with BIOPOLYMER and DISCOVER of a molecular simulation system, InsightII (Version 97.2) (Biosym Technologies Inc.). The coordinates of a retinol-binding protein (*41*), whose Protein Data Bank code is 1rbp, was used as a template for the model of PGDS. The model structure of the protein was energetically optimized by molecular mechanical calculation with DISCOVER. The structure of bilirubin was constructed with BIOPOLYMER. To insert the bilirubin into the model of the protein, first we superimposed the model structure on the structure of bilin-binding protein complexed with a bilin molecule (Protein Data Bank code 1bbp; *42*). Then, the bilirubin was placed in the model structure of the enzyme so that bilirubin was overlaid with bilin in the superimposed bilin-binding protein. The model of the complex structure was energetically optimized again with DISCOVER.

RESULTS

Fluorescence Quenching of PGDS by Bile Pigments and Thyroid Hormones. Several members of the lipocalin superfamily, such as RABP (*43*), LG (*44*−*46*), and plasma retinol-binding protein (*47*), show quenching of their tryptophan fluorescence when bound to their respective ligands. PGDS also shows quenching of its intrinsic tryptophan fluorescence when bound to retinoic acid or retinal but not to retinol (*26*). Therefore, we first examined the fluorescence quenching after incubation of Ala^{65}-PGDS with various hydrophobic substances including bile pigments, thyroid hormones, iodinated amino acids, steroid hormones, and fatty acids. Biliverdin, bilirubin, T4, reverse T3, and T3 induced the fluorescence quenching of the recombinant protein in a concentration-dependent manner, decreasing the fluorescence to 0−10% with biliverdin and bilirubin and to 20−40% with thyroid hormones (Figure 1A). Progesterone also reduced the fluorescence of PGDS to 65% of its initial value (data not shown). T2, 3,5-diiodo-L-tyrosine, 3-iodo-L-tyrosine, testosterone, pregnenolone, estradiol, and docosahexaenoic acid did not cause any fluorescence quenching. The dissociation constants K_d and number of binding sites of Ala^{65}-PGDS were calculated from the fluorescence quenching curves to be 33 nM and 1.03 for biliverdin, 37 nM and 1.08 for bilirubin, 660 nM and 2.12 for T4, 820 nM and 1.32 for reverse T3, and 2.08 μM and 1.66 for T3, respectively (Table 1). When Ala^{89,186}-PGDS was used, the fluorescence quenching was also observed with biliverdin, bilirubin, T4, reverse T3, and T3, giving essentially the same quenching curves as those with the Ala^{65} mutant (data not shown).

Solid-Phase Assay for Binding between PGDS and Lipophilic Ligands. The binding of biliverdin, bilirubin, and

Table 1: Kinetic Constants for Binding of Biliverdin, Bilirubin, and Thyroid Hormones to PGDS[a]

method	parameters	biliverdin	bilirubin	T4	reverse T3	T3
Levine evaluation	K_d (nM)	99	113	660	820	2080
	n	1.03	1.02	2.12	1.32	1.66
Cogan evaluation	K_d	33	37			
	n	1.03	1.08			
IAsys	K_d (nM)	30	65	490	840	4900
	k_a (M^{-1} s^{-1})	9.8×10^2	2.3×10^3	1.4×10^4	1.8×10^4	1.7×10^4
	k_d (s^{-1})	2.9×10^{-5}	1.5×10^{-4}	6.9×10^{-3}	1.6×10^{-2}	8.6×10^{-2}
BIAcore	K_d (nM)			1200	550	730
	k_a (M^{-1} s^{-1})			2.0×10^3	9.5×10^3	9.4×10^3
	k_d (s^{-1})			7.0×10^{-3}	7.2×10^{-3}	1.3×10^{-2}
inhibition	IC_{50} (μM)	5.3	6.8	3.9	9.3	10.9
	K_i (μM)			23		

[a] Fluorescence quenching data were analyzed according to Levine (*33*) and Cogan et al. (*34*). The binding of thyroid hormones was too weak to permit calculation of binding parameters according to Cogan. Abbreviations: K_d, dissociation constant; n, binding ratio of ligand to protein; k_a, association rate constant; k_d, dissociation rate constant; IC_{50}, concentration where 50% of enzyme activity is inhibited; K_i, dissociation constant of enzyme−inhibitor complex according to Michaelis−Menten kinetics; T4, L-thyroxine; reverse T3, 3,3′,5′-triiodo-L-thyronine; T3, 3,3′,5-triiodo-L-thyronine.

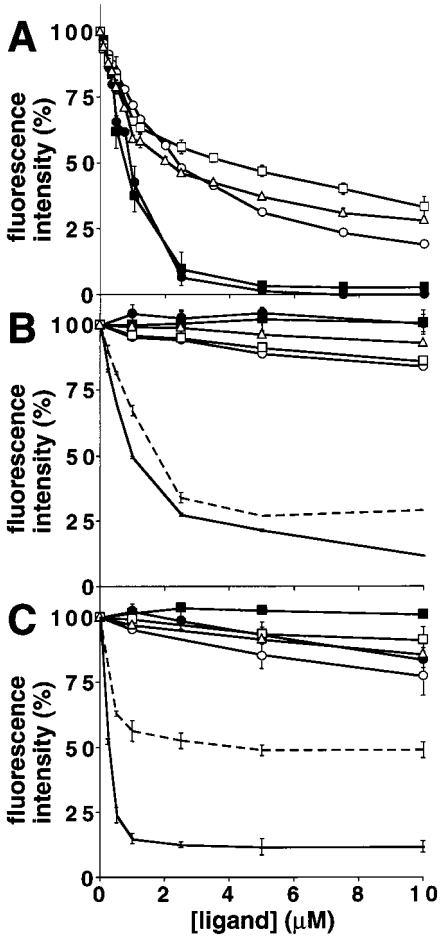

FIGURE 1: Fluorescence quenching behavior of Ala[65]-PGDS, LG, and RABP. (A) Ala[65]-PGDS, (B) LG, and (C) RABP (all 1.5 μM) were incubated with increasing concentrations of bilirubin (closed circles), biliverdin (closed squares), T4 (open circles), T3 (open squares), reverse T3 (open triangles), *all-trans*-retinoic acid (solid line), and *all-trans*-retinol (dashed line); $n = 3-4$; standard errors. In the absence of ligands LG showed 47% and RABP showed 41% of the PGDS fluorescence intensity, respectively.

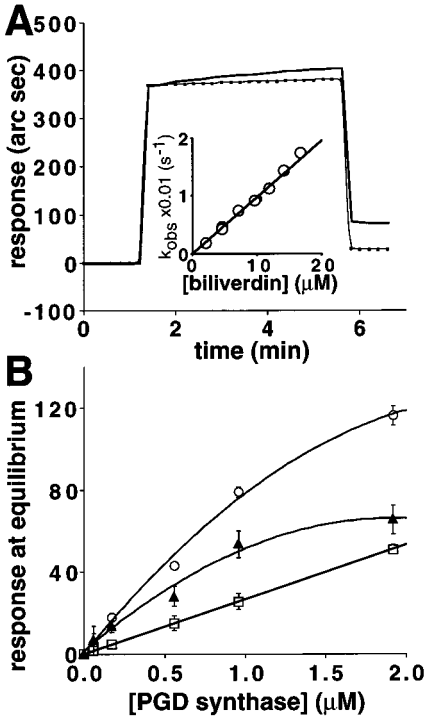

FIGURE 2: Solid-phase assays for binding between Ala[65]-PGDS and lipophilic ligands. (A) Typical time courses of the binding responses evoked by 15 μM biliverdin: LG (dotted line) and Ala[65]-PGDS (solid line) immobilized on sensor surfaces. Inset: plot of pseudo-first-order approximation of binding responses against biliverdin concentration in order to calculate the binding constant K_d. (B) Concentration dependency of Ala[65]-PGDS binding to immobilized reverse T3. Specific binding responses (closed triangles) were calculated from measured total binding responses (open circles) and measured nonspecific binding responses (open squares). The protein concentration where half of the maximum specific binding response occurred was taken to be the dissociation constant. $n = 3$; standard errors.

thyroid hormones to immobilized Ala[65]-PGDS was determined by the resonant mirror technique. A typical time course of the binding of biliverdin is shown in Figure 2A. The K_d values of the immobilized PGDS were determined to be 30 nM for biliverdin, 65 nM for bilirubin, 490 nM for T4, 840 nM for reverse T3, and 4.9 μM for T3, respectively (Table 1). The association rate constants (k_a) were determined from the time course of binding to be 9.8×10^2 M^{-1} s^{-1} for biliverdin, 2.3×10^3 M^{-1} s^{-1} for bilirubin, 1.4×10^4 M^{-1} s^{-1} for T4, 1.8×10^4 M^{-1} s^{-1} for reverse T3, and 1.7×10^4 M^{-1} s^{-1} for T3. The dissociation rate constants (k_d) were calculated from the K_d and k_a values to be 2.9×10^{-5} s^{-1} for biliverdin, 1.5×10^{-4} s^{-1} for bilirubin, 6.9×10^{-3} s^{-1} for T4, 1.6×10^{-2} s^{-1} for reverse T3, and 8.6×10^{-2} s^{-1} for T3 (Table 1).

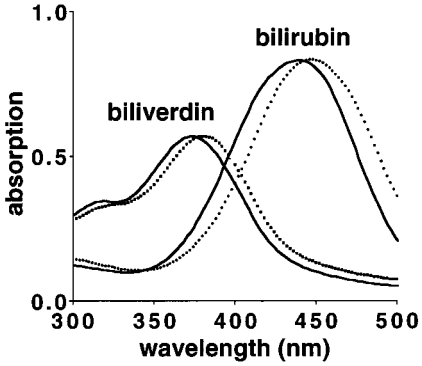

FIGURE 3: Red shift of the absorption spectra of bilirubin and biliverdin upon binding to Ala^{65}-PGDS. Spectra were recorded in the absence (solid line) or presence (dotted line) of an equimolar amount of Ala^{65}-PGDS (15 μM).

The binding of Ala^{65}-PGDS to immobilized thyroid hormones was examined by the affinity-in-solution method with the BIAcore device. The total, nonspecific, and calculated specific binding curves of Ala^{65}-PGDS to immobilized reverse T3 are shown in Figure 2B. The K_d values of Ala^{65}-PGDS for the immobilized ligands were 1.2 μM, 550 nM, and 730 nM for T4, reverse T3, and T3, respectively (Table 1). The k_a values were calculated from the binding curves to be 2.0×10^3 M^{-1} s^{-1}, 9.5×10^3 M^{-1} s^{-1}, and 9.4×10^3 M^{-1} s^{-1} and the k_d to be 7.0×10^{-3}, 7.2×10^{-3}, and 1.3×10^{-2} for T4, reverse T3, and T3, respectively (Table 1).

No significant binding was observed with other ligands, as examined by the solid-phase binding assays with the resonant mirror technique or surface plasmon resonance detection or by the method of Descalzi Cancedda et al. (*40*) (data not shown).

Selectivity of Bile Pigments and Thyroid Hormone Binding among Lipocalins. When two other lipocalins, RABP and LG, were incubated with biliverdin, bilirubin, T4, reverse T3, and T3, no significant quenching of the intrinsic fluorescence was observed up to their concentrations of 10 μM, although *all-trans*-retinoic acid and *all-trans*-retinol caused significant fluorescence quenching (Figure 1B,C). Both RABP and LG also failed to show any significant binding in the solid-phase binding assays (data not shown). These results indicate that PGDS specifically binds bile pigments and thyroid hormones among the three lipocalins.

Photometric Analyses of Bile Pigments and Thyroid Hormones Bound to PGDS. When we recorded absorption spectra of biliverdin and bilirubin in the absence and presence of an equimolar amount of Ala^{65}-PGDS, their absorption peaks shifted to longer wavelengths from 375 to 380 nm and from 439 to 446 nm, respectively, after binding to Ala^{65}-PGDS (Figure 3). However, we could not detect the shift of the spectra of bound thyroid hormones, because their absorption spectra overlapped with the absorption spectrum of PGDS.

CD spectra of bile pigments and thyroid hormones appeared after incubation with Ala^{65}-PGDS in a range of absorption wavelengths above 300 nm for each ligand. Bilirubin bound to PGDS showed a remarkable bisignate CD spectrum with a (−) Cotton effect at 422 nm and a (+) Cotton effect at 472 nm due to two dipyrrinone π-chromophores within one molecule (Figure 4A). The CD spectrum was similar to that of a right-handed chirality of the bilirubin molecule bound to albumin, which showed a minimum at 415 nm and a maximum at 467 nm (*48*). In contrast, biliverdin showed a monosignate (−) Cotton effect with a peak wavelength between 366 and 372 nm due to only one chromophoric π-electronic system extending through the entire molecule (Figure 4B). T4 and reverse T3 showed small, but significant, (+) Cotton effects with peaks at 328 and 330 nm, respectively (Figure 4C). No CD spectrum was detected with T3 under our experimental conditions because of the weak affinity of PGDS for T3. In solution, neither those bile pigments nor thyroid hormones showed CD spectra due to undisturbed rotation. The Ala^{65} mutant also did not show any CD signal in a wavelength range above 300 nm.

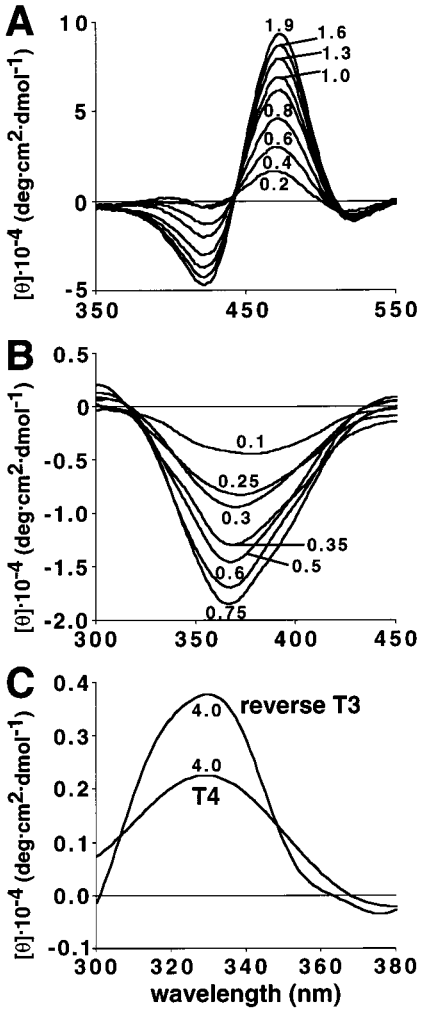

FIGURE 4: CD spectra of lipophilic ligands bound to Ala^{65}-PGDS: (A) bilirubin, 20 nmol; PGDS, up to 38 nmol; (B) biliverdin, 20 nmol; PGDS, up to 15 nmol; (C) T4 and reverse T3, 20 nmol; PGDS, 80 nmol. Numbers indicate the molar ratios of protein to ligand.

These results, taken together, indicate that those hydrophobic ligands were captured by the protein to restrict their movement.

Inhibition of PGDS Activity by Bile Pigments and Thyroid Hormones. T4, biliverdin, bilirubin, reverse T3, and T3 inhibited the PGDS activity of the Ala^{89,186}-PGDS in a concentration-dependent manner (Figure 5A), giving IC_{50} values of 3.9, 5.3, 6.8, 9.3, and 10.9 μM, respectively (Table 1). T2, 3,5-diiodo-L-tyrosine, 3-iodo-L-tyrosine, testosterone, pregnenolone, progesterone, estradiol, and docosahexaenoic

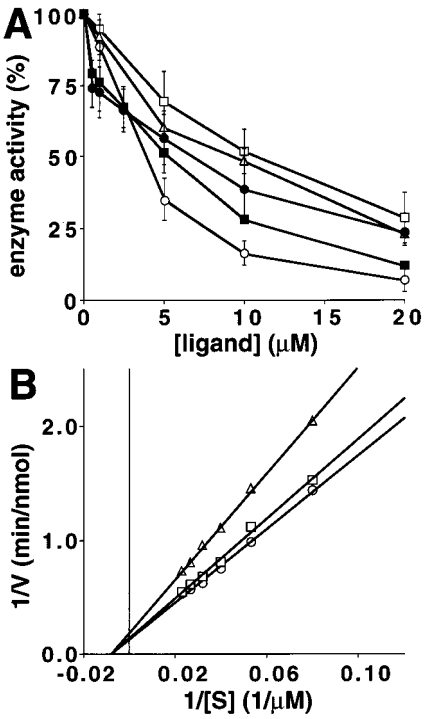

FIGURE 5: Inhibition of $Ala^{89,186}$-PGDS (610 nM) activity by lipophilic ligands: (A) bilirubin (closed circles), biliverdin (closed squares), T4 (open circles), T3 (open squares), and reverse T3 (open triangles); $n = 4$; standard errors. Full enzyme activity corresponds to a turnover of 3 μmol of PGH_2 min^{-1} (mg of protein)$^{-1}$ (B) Lineweaver−Burk plot obtained from activity assays using PGH_2 (10−35 μM) as substrate, in the absence (circles) or presence of 2 μM (squares) or 5 μM (triangles) T4.

acid did not affect the enzyme activity at concentrations up to 20 μM (data not shown). As judged by the IC_{50} values, biliverdin, bilirubin, and thyroid hormones are more potent inhibitors for PGDS than quadrivalent selenium compounds which were previously the only known inhibitors for PGDS, having an IC_{50} value of 12 μM (*49*). As observed in a Lineweaver−Burk plot (Figure 5B), the most effective inhibitory ligand, T4, inhibited PGDS in a noncompetitive manner with a K_i value of 23 μM. Therefore, thyroid hormones and PGH_2 are not considered to compete for the same binding site in PGDS, similar to the case for retinoids (*26*).

DISCUSSION

Lipocalin-Type PGDS Binds Lipophilic Ligands Selectively. In this study, we have demonstrated by several independent approaches that lipocalin-type PGDS binds bile pigments and thyroid hormones. The binding was strongest for biliverdin and bilirubin, whereas thyroid hormone binding was substantially weaker. On the other hand, association and dissociation events between PGDS and ligand were about 6−3000 times faster for thyroid hormones than for bile pigments. The higher speed of association into and dissociation from the hydrophobic pocket of the protein is considered to be due to the smaller overall size of thyroid hormones compared with bile pigments. Although thyroid hormones have a higher molecular weight because they contain heavy iodine atoms, they are smaller than the bile pigments, because these iodine atoms have only the size of a methyl group.

Other lipophilic substances surveyed, including iodinated amino acids, steroid hormones, or fatty acids, did not show

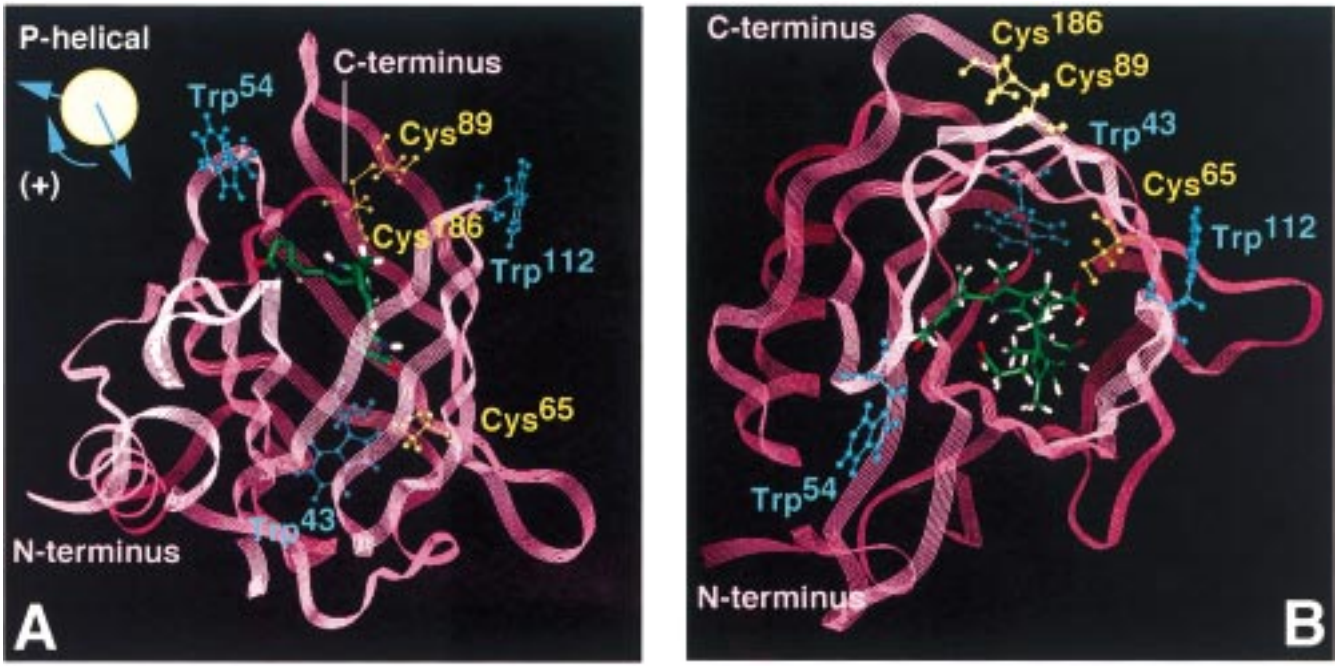

FIGURE 6: Model for binding of bilirubin to lipocalin-type PGDS. The amino acid backbone is shown as a pink ribbon not displaying the side chains. Six amino acid residues are highlighted as ball-and-stick models, Trp^{43}, Trp^{54}, and Trp^{112} in light blue and Cys^{65}, Cys^{89}, and Cys^{186} in yellow. Bilirubin is displayed as a stick model, with carbon atoms in green, nitrogen atoms in dark blue, oxygen atoms in red, and hydrogen atoms in white. Bright colors indicate atoms toward the viewer's position and darker colors those farther away. (A) View from the side of the protein: the entrance to the hydrophobic ligand binding pocket is from the top. For better plasticity, the side chains of bilirubin have been removed, the one dipyrrinone arm of bilirubin containing carbon atoms C1−C9 points toward the viewer, and the other arm containing C11−C19 lies in the paper plane. Bilirubin binds into the pocket with a right-handed chirality, which is also called the P-helical conformation (*62*). (B) View from the top along the ligand binding pocket of PGDS: bilirubin is shown as a complete molecule, with the two dipyrrinone arms in the paper plane.

any binding to PGDS. This demonstrates the selectivity of the protein toward ligands, which is likely to have its basis in the molecular shape of the internal hydrophobic binding pocket of the protein.

Homology Model of Lipocalin-Type PGDS. On the basis of the crystallographic structures of retinol-binding protein (*41*) and bilin-binding protein (*42*), we calculated a three-dimensional model of lipocalin-type PGDS (Figure 6). This model displayed the typical β-sheet barrel structure of lipocalin proteins, where eight β-strands fold around a core cavity, which can bind hydrophobic ligands. In our model of PGDS, Trp^{54} and Trp^{112} were located at the entrance of the cavity, whereas Trp^{43} was buried deeply at the bottom of the pocket. The active center Cys^{65} faced toward the hydrophobic cavity, and Cys^{89} formed a disulfide bridge with Cys^{186}. Bilirubin was fitted into the hydrophobic pocket of the protein (Figure 6) in the energetically most favorable conformation. Biliverdin also showed essentially the same binding conformation (data not shown).

Our CD data of bilirubin binding to PGDS clearly indicated a right-handed chirality of the bilirubin molecule. Figure 6A shows bilirubin in a right-handed conformation in the center of the binding pocket of PGDS.

The fluorescence quenching caused by the ligands can also be explained by our binding model. Bilirubin is able to interact with all three tryptophans contained in PGDS (Figure 6). To clarify which tryptophans contribute to the observed fluorescence, we performed fluorescence quenching assays between mouse Ala^{65}-PGDS and bilirubin, biliverdin, and T4. Mouse PGDS possesses only two tryptophans, at positions 43 and 54, and lacks Trp^{112} at the entrance of the pocket. The fluorescence level of mouse Ala^{65}-PGDS in the absence of lipophilic ligands was 32% of that of the rat Ala^{65}-PGDS. However, after incubation with a 10 μM concentration of the respective lipophilic ligands, the fluorescence decreased to the same level in both proteins (data not shown). This shows that Trp^{112} of the rat protein interacts with bound ligands. We therefore conclude that two or three tryptophan residues in rat Ala^{65}-PGDS contribute to the observed fluorescence of the protein and that the bile pigments interact with these tryptophans, causing a disappearance of fluorescence. Thyroid hormones are likely to interact with one tryptophan residue less in rat Ala^{65}-PGDS, resulting only in a partial quenching of fluorescence.

The model also explains how lipophilic ligands inhibit the enzyme activity. The active Cys^{65} residue is located inside the ligand binding pocket (Figure 6A). Any ligand located in this pocket, therefore, would effectively restrict access of the substrate PGH_2 to the active site.

Specificity of Ligand Binding among Three Lipocalins. Lipocalin-type PGDS is the only enzyme among almost 100 members of the lipocalin family and is recognized as a useful lead molecule for designing new functional proteins (*50*). When we compared the binding behavior of PGDS with that of two other lipocalins, RABP and LG, these two proteins failed to show any significant interaction with bilirubin, biliverdin, and thyroid hormones, as judged by fluorescence quenching assay (Figure 1B,C) and solid-phase binding assays (data not shown). RABP binds *all-trans*- and 13-*cis*-retinoic acid. Interestingly, we found that *all-trans*-retinol also binds to RABP, although the opposite was described earlier (*51*). LG binds a variety of natural ligands such as myristic, palmitic, and oleic acids (*52, 53*) and also retinoic acid (*54*). We found that only PGDS, but not LG and RABP, binds thyroids and bile pigments, although all three of these lipocalins bind retinoids. These results suggest that the size and/or shape of the hydrophobic pocket of PGDS is different from that of the other two lipocalins whose crystal structures have been identified. These data will be useful for designing new functional proteins, which bind and transport hydrophobic xenobiotics, by molecular engineering of lipocalin-type PGDS and other lipocalins.

Physiological Relevance of Binding of Bile Pigments and Thyroid Hormones to PGDS. We demonstrated here that the affinities of PGDS for bile pigments (K_d = 33 and 37 nM) are higher than those for retinoids (K_d = 70−80 nM). These results suggest a novel physiological function of PGDS (β-trace) in the CSF; i.e., this protein may act as a scavenger for hydrophobic harmful compounds produced in the CNS under pathological conditions or invading the CNS from the blood in the case of brain hemorrhages or blood-brain barrier failure. It is in agreement with a previous report by Link and Olsson (*55*) that the CSF level of β-trace was elevated in patients with cerebrovascular lesions. We also confirmed their results by using capillary electrophoretic analysis (*56*).

We extended our studies to search for other nonsubstrate ligands for PGDS and found that the enzyme also specifically binds thyroid hormones. Thyroid hormones are essential for maturation of the CNS. Hypothyroidism results in severe abnormality of the CNS. Transthyretin has been believed to be the sole transporter for thyroid hormones in the CNS. However, no abnormality in the CNS was found in transthyretin-null mutated mice (*57*), suggesting the existence of another thyroid transporter in the CNS. Thus, our findings suggest that PGDS may function as a rescue transporter for thyroid hormones in the mammalian CNS. Although the affinities of PGDS for thyroids are rather weak, the molar concentration of β-trace in the CSF (1 μM) is higher than that of transthyretin (0.3 μM; *24*). On the other hand, PGDS was found to be one of the major proteins that was downregulated in the CNS by hypothyroidism (*58*). We and others recently identified a thyroid-responsive element within the gene for PGDS (*59, 60*). These results also suggest the possible involvement of PGDS in the thyroid-transporting system in the CNS. Transthyretin is produced by the choroid plexus of mammals, birds, and amphibia and secreted into their CSF. However, the amphibian choroid plexus does not produce transthyretin but secretes a lipocalin-type protein (*61*). We recently identified the amphibian choroid plexus protein to be PGDS (unpublished results). Therefore, PGDS may act as a major thyroid transporter in the amphibian CNS.

ACKNOWLEDGMENT

We thank Dr. F. Descalzi Cancedda for kindly performing the assays for binding between PGDS and fatty acids, Mr. D. Irikura for preparation of recombinant PGDS expression vectors, Dr. T. Tanaka for the generous gift of rat recombinant RABP, Dr. G. Schreiber for helpful discussion in preparing the manuscript, Drs. Y. Ishizuka and B. Specht for helpful discussion regarding CD spectroscopy and surface plasmon resonance measurements, respectively, and Ms. S. Sakae and Ms. M. Yamaguchi for kind secretarial assistance.

REFERENCES

1. Urade, Y., Fujimoto, N., and Hayaishi, O. (1985) *J. Biol. Chem. 260*, 12410−12415.
2. Urade, Y., Watanabe, K., and Hayaishi, O. (1995) *J. Lipid Mediators Cell Signalling 12*, 257−273.
3. Urade, Y., and Hayaishi, O. (1999) *Biochim. Biophys. Acta 1436*, 606−615.
4. Ujihara, M., Urade, Y., Eguchi, N., Hayashi, H., Ikai, K., and Hayaishi, O. (1988) *Arch. Biochem. Biophys. 260*, 521−531.
5. Urade, Y., Kitahama, K., Ohishi, H., Kaneko, T., Mizuno, N., and Hayaishi, O. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9070−9074.
6. Beuckmann, C. T., Gordon, W. C., Kanaoka, Y., Eguchi, N., Marcheselli, V. L., Gerashchenko, D. Y., Urade, Y., Hayaishi, O., and Bazan, N. G. (1996) *J. Neurosci. 16*, 6119−6124.
7. Blödorn, B., Mäder, M., Urade, Y., Hayaishi, O., Felgenhauer, K., and Brück, W. (1996) *Neurosci. Lett. 209*, 117−120.
8. Yamashima, T., Sakuda, K., Tohma, Y., Yamashita, J., Oda, H., Irikura, D., Eguchi, N., Beuckmann, C. T., Kanaoka, Y., Urade, Y., and Hayaishi, O. (1997) *J. Neurosci. 17*, 2376−2382.
9. Gerashchenko, D. Y., Beuckmann, C. T., Marcheselli, V. L., Gordon, W. C., Kanaoka, Y., Eguchi, N., Urade, Y., Hayaishi, O., and Bazan, N. G. (1998) *Invest. Ophthalmol. Visual Sci. 39*, 198−203.
10. Tokugawa, Y., Kunishige, I., Kubota, Y., Shimoya, K., Nobunaga, T., Kimura, T., Saji, F., Murata, Y., Eguchi, N., Oda, H., Urade, Y., and Hayaishi, O. (1998) *Biol. Reprod. 58*, 600−607.
11. Gerena, R. L., Irikura, D., Urade, Y., Eguchi, N., Chapman, D. A., and Kilian, G. J. (1998) *Biol. Reprod. 58*, 826−833.
12. Eguchi, Y., Eguchi, N., Oda, H., Seiki, K., Kijima, Y., Matsuura, Y., Urade, Y., and Hayaishi, O. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 14689−14694.
13. Nagata, A., Suzuki, Y., Igarashi, M., Eguchi, N., Toh, H., Urade, Y., and Hayaishi, O. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4020−4024.
14. Peitsch, M. C., and Boguski, M. S. (1991) *Trends Biochem. Sci. 16*, 363.
15. Toh, H., Urade, Y., and Tanabe T. (1992) *Mediators Inflammation 1*, 223−233.
16. Pervaiz, S., and Brew, K. (1987) *FASEB J. 1*, 209−214.
17. Flower, D. R. (1996) *Biochem. J. 318*, 1−14.
18. Kuruvilla, A. P., Hochwald, G. M., Ghiso, J., Castaño, E. M., Pizzolato, M., and Frangione, B. (1991) *Brain Res. 565*, 337−340.
19. Zahn, M., Mäder, M., Schmidt, B., Bollensen, E., and Felgenhauer, K. (1993) *Neurosci. Lett. 154*, 93−95.
20. Hoffmann, A., Conradt, H. S., Gross, G., Nimtz, M., Lottspeich, F., and Wurster, U. (1993) *J. Neurochem. 61*, 451−456.
21. Watanabe, K., Urade, Y., Mäder, M., Murphy, C., and Hayaishi, O. (1994) *Biochem. Biophys. Res. Commun. 203*, 1110−1116.
22. Clausen, J. (1961) *Proc. Soc. Exp. Biol. Med. 107*, 170−172.
23. Hochwald, G. M., and Thorbecke, G. J. (1962) *Proc. Soc. Exp. Biol. Med. 109*, 91−95.
24. Thompson, E. J. (1988) in *The CSF proteins: a biochemical approach*, Elsevier Science Publishers B.V., Amsterdam.
25. Christ-Hazelhof, E., and Nugteren, D. H. (1982) *Methods Enzymol. 86*, 77−84.
26. Tanaka, T., Urade, Y., Kimura, H., Eguchi, N., Nishikawa, A., and Hayaishi, O. (1997) *J. Biol. Chem. 272*, 15789−15795.
27. Urade, Y., Tanaka, T., Eguchi, N., Kikuchi, M., Kimura, H., Toh, H., and Hayaishi, O. (1995) *J. Biol. Chem. 270*, 1422−1428.
28. McPhee, F., Caldera, P. S., Bemis, G. W., McDonagh, A. F., Kuntz, I. D., and Craik, C. S. (1996) *Biochem. J. 320*, 681−686.
29. McDonagh, A. F., and Assisi, F. (1971) *FEBS Lett. 18*, 315−317.
30. Gemmil, C. L. (1955) *Arch. Biochem. Biophys. 54*, 359−367.
31. Edelhoch, H. (1962) *J. Biol. Chem. 237*, 2778−2787.
32. Chen, L. X., Zhang, Z., Scafonas, A., Cavalli, R. C., Gabriel, J. L., Soprano, K. J., and Soprano, D. R. (1995) *J. Biol. Chem. 270*, 4518−4525.
33. Levine, R. L. (1977) *Clin. Chem. 23*, 2292−2301.
34. Cogan, U., Kopelman, M., Mokady, S., and Shinitzky, M. (1976) *Eur. J. Biochem. 65*, 71−78.
35. Nunomura, W., Takakuwa, Y., Tokimitsu, R., Krauss, S. W., Kawashima, M., and Mohandas, N. (1997) *J. Biol. Chem. 272*, 30322−30328.
36. Hall, D. R., Gorgani, N. N., Altin, J. G., and Winzor, D. J. (1997) *Anal. Biochem. 253*, 145−155.
37. Johnsson, B., Löfås, S., and Lindquist, G. (1991) *Anal. Biochem. 198*, 268−277.
38. Karlsson, R. (1994) *Anal. Biochem. 221*, 142−151.
39. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
40. Descalzi Cancedda, F., Malpeli, M., Gentili, C., Di Marzo, V., Bet, P., Carlevaro, M., Cermelli, S., and Cancedda, R. (1996) *J. Biol. Chem. 271*, 20163−20169.
41. Cowan, S. W., Newcomer, M. E., and Jones, T. A. (1990) *Proteins 8*, 44−61.
42. Huber, R., Schneider, M., Mayr, I., Müller, R., Deutzmann, R., Suter, F., Zuber, H., Falk, H., and Kayser, H. (1987) *J. Mol. Biol. 198*, 499−513.
43. Newcomer, M. E., Pappas, R. S., and Ong, D. E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9223−9227.
44. Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Findlay, J. B. C., Sivaprasadarao, R., Jones, T. A., Newcomer, M. E., and Kraulis, P. J. (1986) *Nature 324*, 383−385.
45. Monaco, H. L., Zanotti, G. M., Spadon, P., Bolognesi, M., Sawyer, L., and Eliopoulos, E. E. (1987) *J. Mol. Biol. 197*, 695−706.
46. Cho, Y., Batt, C. A., and Sawyer, L. (1994) *J. Biol. Chem. 269*, 11102−11107.
47. Zanotti, G., Malpeli, G., and Berni, R. (1993) *J. Biol. Chem. 268*, 24873−24879.
48. Lightner, D. A., Gawronski, J. K., and Wijekoon, W. M. D (1987) *J. Am. Chem. Soc. 109*, 6354−6362.
49. Islam, F., Watanabe, Y., Morii, H., and Hayaishi, O. (1991) *Arch. Biochem. Biophys. 289*, 161−166.
50. Toh, H., Kubodera, H., Nakajima, N., Sekiya, T., Eguchi, N., Tanaka, T., Urade, Y., and Hayaishi, O. (1996) *Protein Eng. 9*, 1067−1082.
51. Ong, D. E., and Chytil, F. (1988) *Arch. Biochem. Biophys. 267*, 474−478.
52. Díaz de Villegas, M. C., Oria, R., Sala, F. J., and Calvo, M. (1987) *Milchwissenschaft 42*, 357−358.
53. Pérez, M. D., Díaz de Villegas, C., Sánchez, L., Aranda, P., Ena, J. M., and Calvo, M. (1989) *J. Biochem. 106*, 1094−1097.
54. Fugate, R. D., and Song, P. S. (1980) *Biochim. Biophys. Acta 625*, 28−42.
55. Link, H., and Olsson, J.-E. (1972) *Acta Neurol. Scand. 48*, 57−68.
56. Hiraoka, A., Arato, T., Tominaga, I., Eguchi, N., Oda, H., and Urade, Y. (1997) *J. Chromatogr. 697*, 141−147.
57. Episkopou, V., Maeda, S., Nishiguchi, S., Shimada, K., Gaitanaris, G. A., Gottesman, M. E., and Robertson, E. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2375−2379.
58. García-Fernández, L. F., Rausell, E., Urade, Y., Hayaishi, O., Bernal, J., and Muñoz, A. (1997) *Eur. J. Neurosci. 9*, 1566−1573.
59. White, D. M., Takeda, T., DeGroot, L. J., Stefansson, K., and Arnason, B. G. W. (1997) *J. Biol. Chem. 272*, 14387−14393.
60. García-Fernández, L. F., Urade, Y., Hayaishi, O., Bernal, J., and Muñoz, A. (1998) *Mol. Brain Res. 55*, 321−330.
61. Achen, M. G., Harms, P. J., Thomas, T., Richardson, S. J., Wettenhall, R. E. H., and Schreiber, G. (1992) *J. Biol. Chem. 267*, 23170−23174.
62. Trull, F. R., Ibars, O., and Lightner, D. A. (1992) *Arch. Biochem. Biophys. 298*, 710−714.

BI990261P