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Fine-tuned broad binding capability of human lipocalin-type prostaglandin D synthase for various small lipophilic ligands

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ABSTRACT

The hydrophobic cavity of lipocalin-type prostaglandin D synthase (L-PGDS) has been suggested to accommodate various lipophilic ligands through hydrophobic effects, but its energetic origin remains unknown. We characterized 18 buffer-independent binding systems between human L-PGDS and lipophilic ligands using isothermal titration calorimetry. Although the classical hydrophobic effect was mostly detected, all complex formations were driven by favorable enthalpic gains. Gibbs energy changes strongly correlated with the number of hydrogen bond acceptors of ligand. Thus, the broad binding capability of L-PGDS for ligands should be viewed as hydrophilic interactions delicately tuned by enthalpy–entropy compensation using combined effects of hydrophilic and hydrophobic interactions.

1. Introduction

The intermolecular interactions of macromolecules with partner molecules represent some of the most fundamental biological processes that include substrate recognition of enzymes, antigen–antibody reactions, and signal transductions [1,2]. To date, several key binding models have been suggested to obtain a general understanding of intermolecular binding mechanisms: the lock and key model, induced fit model, and conformational selection (pre-existing) model [3,4]. These models have complementarily explained various intermolecular interactions based on the static structural shapes and dynamic conformations of interacting sites. However, they have not yet been elucidated in detail molecular origins and mechanisms from a thermodynamic viewpoint. The thermodynamic characterization of binding systems is critical because the formation of all complexes is governed by an energetic balance toward a more favorable state by decreasing the Gibbs energy change (ΔG) [5], which is often difficult to observe using only structure-based approaches. The driving forces for binding have been simply expressed by two terms: an enthalpy change (ΔH) and entropy change (ΔS) in the equilibrium system [6,7]. Enthalpy is an excellent reporter of changes in intermolecular contacts such as electrostatic interactions among charges and/or dipoles, hydrogen bonds, and van der Waals' interactions [2,8,9]. On the other hand, entropy changes come from the dehydration and change in molecular flexibility as a result of altering the degree of freedom of a system [2,10]. Enthalpy and entropy changes occur concomitantly and enthalpy–entropy compensation has been shown to limit binding affinity [6,11]. Furthermore, these driving forces sensitively reflect even subtle changes in molecular conformations and hydrational states or interactions with ions [12–14]. Therefore, isothermal titration calorimetry (ITC) is a powerful approach to explore intermolecular interactions due to the direct and precise observation of binding enthalpy [1].

Abbreviations: PG, prostaglandin; L-PGDS, lipocalin-type prostaglandin D synthase; K_d, dissociation constant; ITC, isothermal titration calorimetry; T4, L-thyroxine; T3, 3,3′,5-triiodo-L-thyronine; TNS, 2-(*p*-toluidinil) naphtalene-6-sulfonic acid

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The selection of proper binding systems is a critical point in characterizing intermolecular interactions using ITC. We recently performed *in vitro* binding studies of human lipocalin-type prostaglandin (PG) D synthase (L-PGDS, prostaglandin-H2 D-isomerase, EC 5.3.99.2) using the tryptophan fluorescence quenching, TNS competition assay and ITC [15]. Spectroscopic studies revealed that Human L-PGDS could bind to a large range of lipophilic binding partners, including heme metabolites, retinoids, thyroids, steroids, flavonoids, and saturated fatty acids, which differ in molecular size and physico-chemical properties [15]. In addition, ITC results showed that L-PGDS bound to two molecules of heme metabolites such as hemin, biliverdin, and bilirubin with high and low affinities [15]. We previously proposed that the two-molecules capturing abilities of L-PGDS for heme metabolites may play an important role in inhibiting the onset of the delayed cerebral vasospasms in patients with subarachnoid hemorrhage [15,16]. Hence, the capability of L-PGDS to bind with various lipophilic ligands is suitable for ITC-based thermodynamic surveys.

L-PGDS is known to be a multi-functional protein that acts as a PGD_2-producing enzyme [17,18], active oxygen scavenger [19,20], and secretory transporter protein for small lipophilic molecules [21]. L-PGDS is also a member of the lipocalin superfamily; lipocalins are small globular proteins of approximately 200 amino acid residues [22–24] that play a role in the storage and transport of small lipophilic molecules such as vitamins and fatty acids [25]. Using these properties of L-PGDS, we recently proposed that the drug delivery system of L-PGDS as a delivery vehicle could facilitate the pharmaceutical development and clinical usage of various water-insoluble compounds [26]. L-PGDS has a typical lipocalin fold that consists of an eight-stranded anti-parallel β-barrel that forms a hydrophobic cavity and long α-helix [23,27], and the hydrophobic cavity encloses the binding sites for small lipophilic ligands [28,29] (Fig. 1A). It has been widely accepted that L-PGDS and other lipocalins take advantage of their hydrophobic cavities to capture lipophilic ligands using hydrophobic interactions. The classical hydrophobic effects due to the dehydration of water molecules surrounding the protein and ligand to bulk have been suggested to dominate the binding of lipophilic ligands in the hydrophobic cavity [30,31]. Although entropy-driven complex formation between L-PGDS and small lipophilic ligands is expected, systematic thermodynamic investigations have not yet been conducted.

To elucidate the relative contributions of driving forces to intermolecular interactions, we investigated the ITC-based thermodynamics of 18 binding systems of L-PGDS with 15 variations of ligands (retinoic acids, L-thyroxine, progesterone, genistein, and PGH_2-analog U-46619) including the 3 heme metabolites (hemin, biliverdin, and bilirubin) previously studied [15]. The results showed that the formation of all complexes was driven by enthalpy mainly due to intermolecular hydrogen bonding, and not always by entropic gains such as the typical concept of the hydrophobic effect. In addition, we proposed that the broad binding capability of L-PGDS resulted from the delicate balance achieved by enthalpy and entropy compensation through hydrophilic and hydrophobic interactions.

2. Materials and methods

2.1. Chemicals

Bilirubin, all-*trans* retinoic acid, 9-*cis* retinoic acid, and corticosterone were purchased from Wako. Hemin, L-thyroxine (T4), 3,3′,5-triiodo-L-thyronine (T3), progesterone, genistein, and daidzein were obtained from Sigma. Biliverdin, testosterone, naringenin, U-46619, and 2-(*p*-toluidinyl) naphthalene-6-sulfonic acid (TNS) were purchased from MP Biomedicals, Fluka, Chroma Dex, Cayman Chemical, and Invitrogen, respectively. All ligands were dissolved in dimethyl sulfoxide (DMSO). The concentrations of hemin, biliverdin, bilirubin, T4, T3, and TNS in DMSO were determined spectroscopically with a molar extinction coefficient described previously [15]. The concentrations of the other ligands were determined by their molecular weights. All chemicals were of analytical grade.

2.2. Purification of recombinant human L-PGDS

The open reading frame for human L-PGDS, which was composed of 190 amino acid residues (GenBank accession No. M61900) [32], was ligated into the BamHI-EcoRI sites of the expression vector pGEX-2T (GE Healthcare Bio-Sciences). The N-terminal 22-amino acid residues corresponding to the putative secretion signal peptide of L-PGDS were truncated. C65A/C167A (ε_{280} = 25 900 $M^{-1} \cdot cm^{-1}$)-substituted L-PGDS was expressed in *Escherichia coli* BL21 (DE3) (TOYOBO) [15]. Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene). The mutated L-PGDS was expressed as a glutathione *S*-transferase fusion protein. The fusion protein was bound to glutathione–Sepharose 4B (GE Healthcare) and incubated overnight with 165 units of thrombin to release L-PGDS. The recombinant proteins were further purified by gel filtration chromatography with HiLoad 16/600 Superdex 75 (GE Healthcare) in 5 mM Tris/HCl buffer (pH 8.0) and were then dialyzed against 50 mM Tris/HCl buffer (pH 8.0), 50 mM HEPES buffer (pH 8.0), or 50 mM sodium phosphate buffer (pH 8.0). In the present study, the C65A/C167A-substituted L-PGDS was employed instead of the wild-type protein, because the amount of the wild type is limited due to the incorrect intra- and intermolecular disulfide bonds and protein aggregation.

2.3. Isothermal titration calorimetry

Calorimetric experiments were performed with a NanoITC instrument (TA instruments) and VP-ITC instrument (MicroCal Inc.) in 50 mM Tris/HCl buffer (pH 8.0), 50 mM HEPES buffer (pH 8.0), and 50 mM sodium phosphate buffer (pH 8.0) containing 5% (v/v) DMSO at 25 °C (Fig. 2 and Supplementary Figs. 1 and 2). L-PGDS (120–1500 μM) in the injection syringe was titrated into 69–86 μM hemin, 35–38 μM biliverdin, 39–41 μM bilirubin, 12–15 μM all-*trans* retinoic acid, 15–27 μM 9-*cis* retinoic acid, 24–30 μM T4, 30–40 μM T3, 30–80 μM progesterone, 40–80 μM testosterone, 80 μM corticosterone, 30–40 μM genistein, 60–65 μM naringenin, 60–65 μM daidzein, 35–40 μM U-46619, and 60–150 μM TNS, respectively. Titration experiments consisted of 25–58 injections spaced at intervals of 300–360 s. The injection volume was 2 μl or 5 μl for each, and the cell was continuously stirred at 270–351 rpm. The corresponding heat of dilution of L-PGDS titrated to the buffer was used to correct the data. Observed enthalpy changes (ΔH_{obs}°) for binding and the dissociation constant (K_d) were directly calculated from the integrated heats using the one-set of independent binding sites model supplied by MicroCal Origin software. The equation of this binding model (Eq. (1)) was:

$$Q = \frac{n[P]_t \Delta H^{\circ}_{obs} V_0}{2}\left[1 + \frac{L_R}{n} + \frac{K_d}{n[P]_t} - \sqrt{\left(1 + \frac{L_R}{n} + \frac{K_d}{n[P]_t}\right)^2 - \frac{4L_R}{n}}\right] \quad (1)$$

where Q is the change in heat in the system, V_0 is the effective volume of the calorimeter cell (1.43 ml for the VP-ITC instrument and 0.17 ml for the NanoITC instrument), L_R is the ratio of the total ligand concentration to total protein concentration ($[P]_t$) at any given