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Quantitation of Thyroid Hormone Binding to Anti-Thyroxine Antibody Fab Fragment by Native Mass Spectrometry

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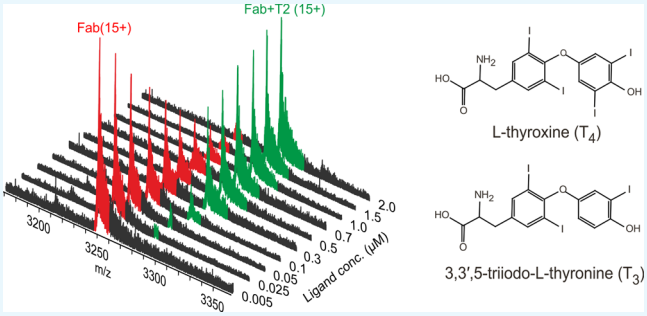
Supporting Information

ABSTRACT: Thyroid hormones are important regulatory hormones, acting on nearly every cell in the body. The two main thyroid hormones are L-thyroxine (tetraiodo-L-thyronine, T_4) and 3,3′,5-triiodo-L-thyronine (T_3), which are produced in the thyroid gland and secreted into the blood stream. Other important thyroid hormone metabolites are 3,3′-diiodo-L-thyronine (T_2) and L-thyronine (T_0), which may show increased levels in circulation due to dietary iodine deficiency or other medical disorders. Owing to their central role in cellular functions, sensitive and specific detection methods for thyroid hormones are needed. In this work, native mass spectrometry (MS) was used to quantitate thyroid hormone binding to the anti-T_4 antibody Fab fragment. First, the binding affinity for T_2 was determined via direct ligand titration experiments. Then, the affinities for the other ligands were determined by competition experiments using T_2 as the "low-affinity" reference ligand. The highest affinity was measured for T_3, followed by T_4, T_2, and T_0 (K_d = 29, 3.4, and 260 nM and 130 μM, respectively). Thus, it is evident that the number and positions of the iodine substituents within the thyronine rings are important for the ligand binding affinity of anti-T_4 Fab. Surprisingly, structurally related tetrahalogen bisphenols were also able to bind to anti-T_4 Fab with nanomolar affinities.



INTRODUCTION

Thyroid hormones (THs) play an important role in the regulation of metabolism, especially growth and development.[1] THs exert their effects by binding to thyroid hormone receptors (THRs), which are members of a nuclear hormone receptor family. THRs exist in three major isoforms, THRα1, THRβ1, and THRβ2.[2]

Thyroid hormones are derivatives of L-tyrosine and contain a variable amount of iodine substituents in their structures (Figure 1). They are produced in the thyroid gland, which secretes these hormones into the blood stream. The most abundant TH in the blood is L-thyroxine (tetraiodo-L-thyronine, T_4), which contains four iodine substituents in the ring positions 3, 5, 3′, and 5′. In the cells, T_4 is converted to a more potent 3,3′,5′-triiodo-L-thyronine (T_3) by deiodinases. The binding affinity of T_3 to THRα1 is significantly stronger (K_d = 0.06 nM) as compared to T_4 (K_d = 2 nM).[3]

In serum, THs exist either free or bound to several different proteins, such as thyroxine-binding globulin, transthyretin, albumin, and apolipoproteins. Concentrations of the protein-bound THs are in the nanomolar level, while free THs are present in picomolar concentrations.[4] The concentration of free T_4 in the blood stream is higher than that of T_3 (typically around 19 and 4 pM).[3]

There are also a number of other compounds that structurally resemble T_4 and T_3. These include thyroid hormone metabolites, 3,3′,5′-triiodo-L-thyronine (so-called reverse T_3), 3,3′-diiodo-L-thyronine (T_2), and L-thyronine (T_0), as well as other compounds such as different organohalogen compounds. One class of such compounds is bisphenols, which are widely used as starting materials in polycarbonate and epoxy resin synthesis. Different bisphenols have also potential to bind different nuclear hormone receptors and may affect a variety of physiological functions.[5,6] Thus, thyroid hormones, their metabolites, and other structurally related compounds (e.g., halogenated bisphenols) form a group of molecules, which may bind to the same receptor protein(s) with different affinities and specificities. Sensitive and specific detection methods for these molecules are therefore needed.

Two widely used, label-free techniques for biomolecular interaction analysis are surface plasmon resonance (SPR)[7] and isothermal titration calorimetry (ITC).[8] In SPR, the other binding partner (usually ligand) is covalently immobilized onto a sensor surface and the binding of the other analyte (protein)

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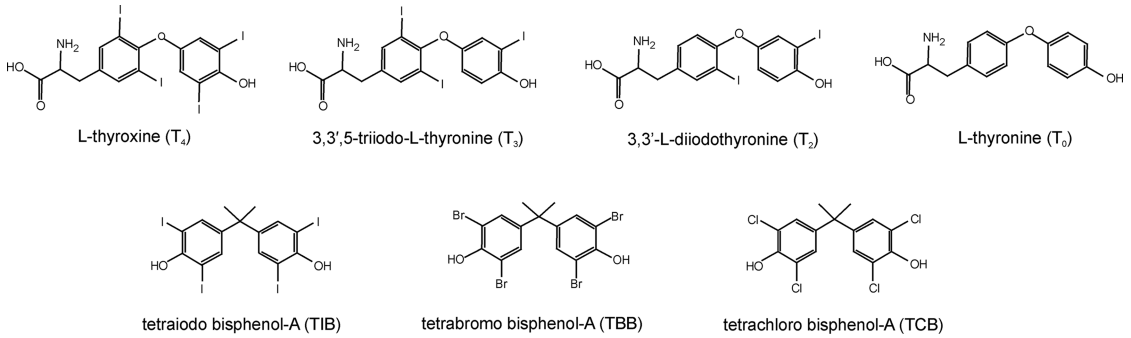

Figure 1. Chemical structures of thyroid hormones and bisphenols used in this study.

is then measured. SPR directly provides association (k_{on}) and dissociation (k_{off}) rate constants for the interaction, which can be used to calculate dissociation constant (K_d) for the binding. In ITC, the biomolecular interaction is quantified by measuring the heat (enthalpy) associated with the binding, which is used to determine K_d as well as other thermodynamic parameters. Therefore, ITC can be considered a true label-free technique. However, ITC suffers from high sample consumption, lengthy analysis times, and low sensitivity, which limits the accessible K_d range. SPR is more sensitive and faster than ITC and also directly provides kinetic information, but the method is not truly label free as one binding partner is immobilized, which may alter binding thermodynamics. Furthermore, neither SPR nor ITC directly provides binding stoichiometry, and therefore, the obtained values depend on the model used for the data fitting.[9]

An alternative method to study ligand binding to proteins is native mass spectrometry (MS).[10] In native MS, proteins are measured in their folded, biologically active states. Native MS does not require any labeling or immobilization of the binding partners, which could have adverse effects on the binding. Furthermore, native MS is more sensitive and faster as compared to many other techniques and can also be used to screen more than one ligand at the time. Unlike any other technique, native MS directly provides the binding stoichiometry since separate signals for the free and bound protein forms are obtained. In addition, measurements can be carried out with very small amounts of purified protein materials and even with endogenous protein samples.[9,11]

In this work, native MS was used to quantitate thyroid hormone binding to the anti-thyroxine antibody Fab fragment (anti-T_4 Fab). To gain insight into the binding specificity of anti-T_4 Fab, we chose T_4, T_3, T_2, and T_0 among different thyroid hormones for this study. In addition, binding of structurally related tetrahalogen bisphenols (tetraiodobisphenol-A, TIB; tetrabromobisphenol-A, TBB; tetrachlorobisphenol-A, TCB) was also characterized (Figure 1). The work was carried out in three steps: (1) initial screening of the ligands to find approximate binding affinities and to select a "low-affinity" reference ligand, (2) direct titration of a reference ligand to determine its accurate K_d value, and (3) ligand competition experiments with the other ligands to determine their respective K_d values. This workflow provided accurate K_d values for all the ligands studied, ranging from low nanomolar to high micromolar levels. The results well demonstrate the performance of native MS in the accurate analysis of ligand binding thermodynamics (at high- and low-affinity regimes) as well as high sensitivity of the technique (i.e., only ~0.5 μg of protein consumed per a single native MS experiment).

RESULTS AND DISCUSSION

MS Analysis of Anti-T_4 Fab Fragment. Prior to the ligand binding experiments, the desalted anti-T_4 Fab fragment was analyzed in both denaturing and native solution conditions (Figure 2). In the denaturing conditions, the electrospray ionization Fourier transform ion cyclotron resonance (ESI FT-ICR) mass spectrum of anti-T_4 Fab (Figure 2A) exhibited a broad charge state distribution (CSD) centered at high charge states (20+ to 42+ at m/z 1200−2200), consistent with the fully denatured (unfolded) protein in these conditions. The most abundant isotopic mass, obtained from the deconvoluted mass spectrum (see inset in Figure 2A), was determined to be 48703.897 Da, which differs from the calculated mass (48643.489 Da) by 60.41 Da. The reason for this deviation is not known but was not further investigated since the protein was able to bind its target ligands (this study). In contrast, the mass spectrum measured in the native solution conditions (Figure 2B) displayed a narrow charge state distribution centered at low charge states (16+ to 13+ at m/z 3000−3500), indicating that anti-T_4 Fab remained fully folded in these conditions.[12] Therefore, 20 mM aqueous ammonium acetate solution (pH 6.9) was selected as the solvent for the further ligand binding experiments. The direct infusion ESI FT-ICR experiments permitted native MS measurements of anti-T_4 Fab even at 10 nM protein concentration (data not shown). However, for the further experiments, the protein concentration was fixed to 0.1 μM to obtain sufficiently high signal-to-noise (S/N) ratios for more accurate binding constant determinations.

Ligand Screening. The initial ligand screening experiments were performed to determine the approximate binding affinities of the ligands toward anti-T_4 Fab. The mass spectra indicated only 1:1 binding for the five ligands (T_4, T_3, T_2, TIB, and TBB), at different ligand concentrations, suggesting that the ligand binding was specific (Figures S1−S7). The only exceptions were T_0 and TCB for which also the binding of the second ligand at the highest ligand concentrations was observed. This most likely represents nonspecific binding to the other than the primary binding site. Based on the initial ligand screening, T_4 and T_3 were recognized as the high-affinity ligands, having low nanomolar binding affinities. In addition, T_2 showed clearly a weaker binding affinity, being in the submicrometer range. The remaining ligands (T_0, TIB, TBB, and TCB) showed submicromolar to micromolar affinity range. To measure the K_d values, direct ligand titration experiments for the high-affinity ligands T_4 and T_3 were not possible at 0.1 μM protein concentration because the free ligand concentration would have been extremely low at any

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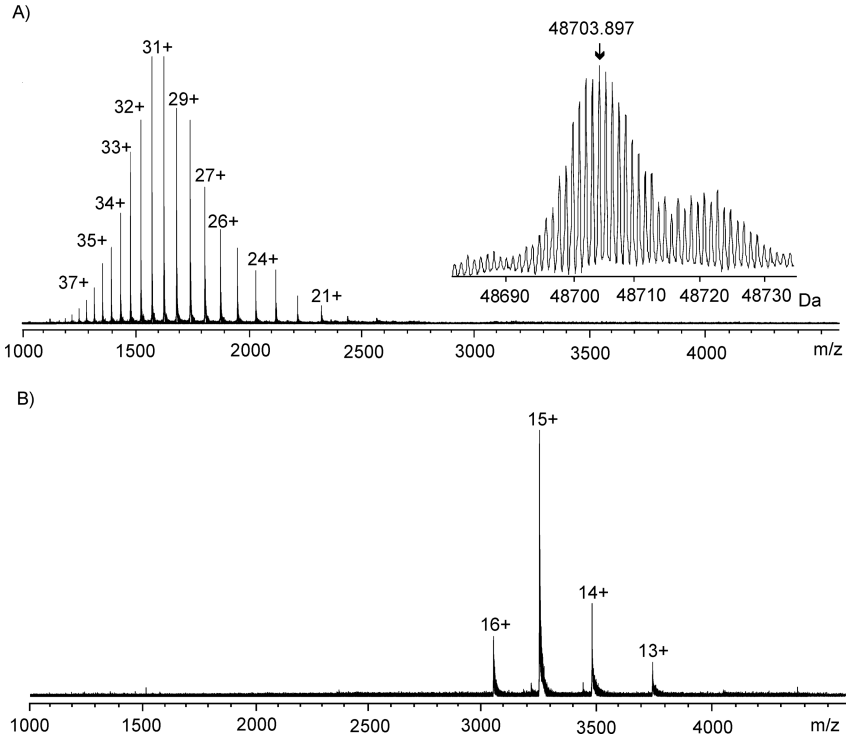

Figure 2. 12-T ESI FT-ICR mass spectra of 0.1 μM of anti-T_4 Fab in (A) $CH_3CN/H_2O/CH_3COOH$ (49.5:49.5:1, v/v; pH 3.2) (denaturing conditions) and (B) 20 mM aqueous ammonium acetate (pH 6.8) (native conditions). In (A), the inset shows the deconvoluted mass spectrum with the peak representing the most abundant isotopic mass marked with an arrow.

substoichiometric ligand concentration, making it difficult to obtain accurate K_d values. Therefore, we decided to first perform direct ligand titration experiments with the "low-affinity" ligand (T_2) against anti-T_4 Fab to obtain its accurate K_d value. Then, this ligand would be used as a reference ligand to find K_d values for the other (low or high affinity) ligands through competitive ligand binding experiments.

Determination of K_d for T_2–Anti-T_4 Fab Complex by Using a Direct Ligand Titration. Based on the initial screening, the T_2 ligand was selected as the "low-affinity" reference ligand. For the titration, 10 different ligand concentrations were used, ranging from 0.005 to 2 μM, at a fixed Fab concentration of 0.1 μM. The native ESI FT-ICR mass spectra showed only the peaks corresponding to the free Fab and the Fab–ligand complex, implying high binding specificity (Figure 3A). From the ESI-MS spectra, we calculated the free and bound protein concentrations using the peak intensities and deduced the free ligand concentrations.[13] The MS titration plot for T_2 binding is presented in Figure 3B. The curve fitting yielded a K_d value for T_2 of (2.63 $\pm$ 0.16) $\times$ 10^{-7} M. We then used this value to determine the dissociation constants for the other ligands by using ligand competition experiments, having at least two different ligand concentrations.

Determination of K_d Values for the Other Ligand–Fab Complexes by Using Ligand Competition Experiments. The T_2 ligand was used as a "low-affinity" reference ligand (L_{ref}) to determine K_d values of the other (high and low affinity) ligands. The representative mass spectra of the ligand competition experiments are presented in Figure 4 (for the other ligands, see Figure S8). The determined K_d values of the thyroid hormones and bisphenols are reported in Table 1. Among the thyroid hormones, T_3 had the highest affinity, followed by T_4, T_2, and T_0. Adamczyk and co-workers studied thyroid hormone binding to anti-T_4 Fab earlier by using SPR.[14,15] They found that T_4 had the highest affinity (approximately 10 times higher than that of T_3) and that T_2 did not show any binding, at least within the K_d range accessible by the SPR technique. The differences in the ligand affinities might well be due to the structural differences between antibodies, which manifest in different specificities. Also, solubilities of ligands and differences in the experimental setup may affect results. In SPR, one component is immobilized, whereas in ESI-MS, both components are in solution.[8] Only T_0 had clearly a weaker binding affinity (approximately five orders of magnitude lower than that of T_3), which suggests that the iodine substituents have a decisive role for the ligand recognition of the anti-T_4 Fab fragment. Interestingly, bisphenols also had relatively high binding affinities toward the Fab fragment with the affinities lowering as the halogen size gets smaller (i.e., I > Br > Cl). It is therefore evident that anti-T_4 Fab is able to accept many different ligands in its binding site (i.e., promiscuous ligand binding). These results are in line with some previous studies.[6]

Additionally, measurements were performed to investigate whether the dissociation constants were independent on the used ligand concentrations. This self-validation experiment was done by using two different concentrations for T_4/T_2, T_3/T_2, and T_0/T_2 ligand pairs (i.e., 0.14/0.5, 0.14/0.5, and 15/0.05 μM, respectively). The determined K_d values were very similar to those determined earlier; K_d for T_4 was (2.39 $\pm$ 0.37) $\times$ 10^{-8} M, K_d for T_3 was (3.80 $\pm$ 0.14) $\times$ 10^{-9} M, and K_d for T_0 was (1.74 $\pm$ 0.18) $\times$ 10^{-4} M. Thus, it is evident that the chosen ligand concentrations were optimal for ligand competition experiments and did not markedly affect the obtained K_d values.

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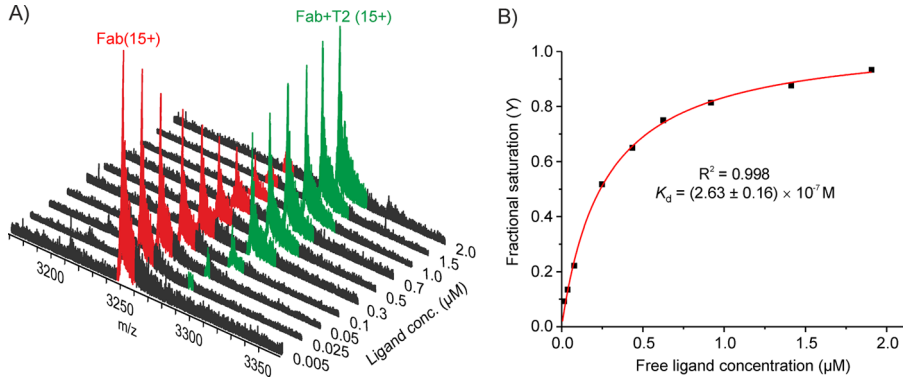

Figure 3. Direct ligand titration of anti-T_4 antibody Fab fragment with 3,3′-diiodo-L-thyronine (T_2). (A) Native ESI FT-ICR mass spectra (15+ charge state) of 0.1 μM anti-T_4 Fab with varying T_2 concentrations measured in 20 mM ammonium acetate (pH 6.8). (B) Titration plot (fractional saturation vs free ligand concentration). Each data point is an average value from the five replicate samples. The solid red line represents the best fit to the specific one-site binding model.

Table 1. Dissociation Constants for Thyroid Hormones and Tetrahalogen Bisphenols with Anti-T_4 Fab Determined by Native MS

ligand	ligand concentration (μM)	reference ligand (T_2) concentration (μM)	K_d (M)[b]	K_d (nM)
T_4	0.07	0.5	$(2.9 \pm 0.4) \times 10^{-8}$	29 ± 4
T_3	0.05	0.5	$(3.4 \pm 0.7) \times 10^{-9}$	3.4 ± 0.7
T_2	0.005–2.0[a]		$(2.6 \pm 0.2) \times 10^{-7}$	260 ± 20
T_0	12	0.07	$(1.3 \pm 0.1) \times 10^{-4}$	130,000 ± 10,000
TIB	0.1	0.7	$(1.1 \pm 0.1) \times 10^{-8}$	11 ± 1
TBB	0.5	1.2	$(8.7 \pm 0.5) \times 10^{-8}$	87 ± 5
TCB	10	0.4	$(7.8 \pm 0.5) \times 10^{-6}$	7800 ± 500

[a]Direct ligand titration; average value from five replicate measurements of two different samples. [b]Average value from 10 replicate measurements of three different samples (except for T_2).

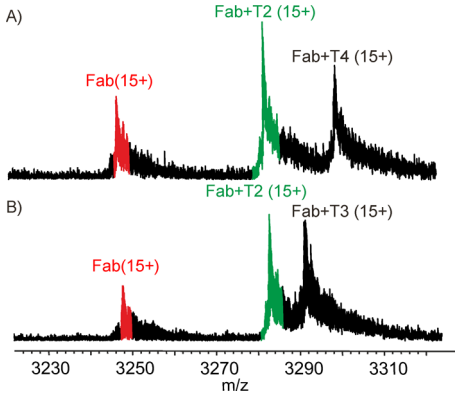

Figure 4. Native 12-T ESI FT-ICR mass spectra of 0.1 μM of anti-T_4 antibody Fab fragment with (A) 0.5 μM 3,3′-diiodo-L-thyronine (T_2) and 0.07 μM L-thyroxine (T_4) and (B) 0.05 μM 3,3′,5′-triiodo-L-thyronine (T_3), measured in 20 mM ammonium acetate (pH 6.8).

CONCLUSIONS

The binding affinities of selected thyroid hormones and related halogenated bisphenols to the anti-T_4 antibody Fab fragment were successfully determined by using a native MS-based method. The results clearly show that the iodine atoms in thyroid hormones play a major role in the ligand binding affinity and selectivity. The ligand without iodine substituents (T_0) had approximately five orders of magnitude lower binding affinity as compared to the ligand with the highest affinity (T_3). The results suggest that the ligand with three iodine atoms (T_3) has even a higher affinity as compared to the ligand with four iodine atoms (T_4). The reason for this is not evident without further structural studies, but it can be noted that T_3 also binds with higher affinity to the thyroid hormone receptor as compared to T_4.[3] On the other hand, T_4 has been reported to have a higher affinity to the plasma membrane receptor on integrin $\alpha v\beta 3$, which initiates nongenomic actions.[16,17] It is also remarkable that two tetrahalogen bisphenols, TIB and TBB, also show nanomolar binding affinities to anti-T_4 Fab, which point out to their potential to bind to other thyroid hormone receptors, which may result in endocrine disrupting activities.[5] As stressed by Jecklin et al, the use of several independent methods is advantageous in determining binding affinities of ligands to proteins.[8,9] The current results show that native MS serves as an effective, independent method to accurately measure dissociation constants for protein–ligand complexes within a large dynamic range (from low nanomolar to millimolar affinity levels) and has some benefits over more conventional techniques, such as a high dynamic range, high sensitivity, and possibility to screen several ligands at the same time, as well as a direct stoichiometrical information directly provided. Native MS could also serve as a promising tool to characterize specificity of closely related ligands such as thyroid hormones to further to clarify cellular events on which thyroid hormone regulation would be a good example.[17]

EXPERIMENTAL SECTION

Materials. The Fab fragment antibody library from a multi-immunized mouse was constructed and displayed to a surface of bacterial phages as described by Tullila and Nevanen.[18] Multi-immunization included thyroxine (T_4) and triiodothyronine (T_3). The anti-T_4 Fab repertoire was enriched utilizing

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streptavidin-coated magnetic beads (SpeedBeads; Thermo Fisher Scientific, Santa Clara, CA) functionalized with a biotinylated T_4-alkaline phosphatase conjugate and a King-Fisher magnetic particle processor (Thermo Fisher Scientific) with a method described by Tullila and Nevanen.[18] After four rounds of selection, a total of 384 single colonies were cultivated and induced for antibody production in 96-well plates. The T_4 binding affinity was measured by an enzyme-linked immunosorbent assay (ELISA). A robotic station (Beckman Coulter) was employed to ELISA screening assays as described by Arola et al.[19] The antibody clone giving the highest intensities against T_4- and T_3-HSA-coated wells in ELISA was chosen for production and purification.

The anti-T_4 Fab-fragment was produced in *Escherichia coli* and purified by using an immobilized metal affinity chromatography followed by a protein G affinity chromatography. The produced protein was analyzed by a nonreducing SDS-PAGE using GelCode staining (Thermo Fisher Scientific) and showed a high level of purity. All thyroid hormones (T_4, T_3, T_2, and T_0), tetrahalogen bisphenols (TIB, TBB, and TCB), and ultrapure ammonium acetate (NH_4OAc; 99.999%) were obtained from Sigma-Aldrich (Saint Louis, MO). Prior to the mass measurements, the protein sample was concentrated by using a 5 kDa MWCO centrifugal filter device (Vivaspin 2; GE Healthcare, Gillingham, U.K.) using ultracentrifugation at 15,000 rpm (Eppendorf 5804 R) at 4 °C. The concentrated protein sample was further desalted with a Sephadex G-25 M (PD-10; GE Healthcare) column, using aqueous ammonium acetate (20 mM; pH 6.8) as an eluent. The protein stock solution concentration was determined by using the Bio-Rad DC protein assay[20,21] with bovine serum albumin as the standard, and the absorbance of the protein sample was determined at 280 nm with a UV spectrophotometer (VWR Spectrophotometer UV-1600PC). All the ligands were accurately weighed and dissolved in 4 M NH_4OH/ethanol (1:1, v/v) to a concentration of 1 mM. All the solvents (HPLC grade) were also purchased from Sigma-Aldrich. The protein and ligand solutions were stored at −20 °C prior to use. The structures of the ligands are shown in Figure 1.

Mass Spectrometry. All mass spectrometric experiments were performed by using a 12-T Bruker solariX XR Fourier transform ion cyclotron resonance (FT-ICR) instrument (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source (Apollo-II). Mass spectra were obtained in a positive ion mode, and the samples were directly infused to the ion source at a flow rate of 2 μL min^{-1}. Dry nitrogen was used as nebulizing (4.0 L min^{-1}) and drying (80 °C, 1.0 bar) gas. The ions were accumulated in the hexapole ion trap for 3.5 s and then transferred to the dynamically harmonized ICR cell (Paracell) for trapping, excitation, and detection. The time of flight was set to 3.5 ms. All other instrumental parameters were carefully adjusted to preserve noncovalent interactions in the gas phase. One hundred 1024 kWord time-domain transients were summed for each spectrum and zero-filled once prior to fast Fourier transform (FFT) and magnitude calculation. The mass spectra were externally calibrated by the ESI Tuning mix (part no. G2431A; Agilent Technologies, Santa Clara, CA). The mass spectra were acquired from m/z 1500 to 5000. The data acquisition was attained by ftmsControl 2.1 software and further processed and analyzed by DataAnalysis 4.2 SR1 software. All native MS experiments were performed in 20 mM aqueous ammonium acetate (pH 6.9) solution. For denaturing MS experiments, a mixture of $CH_3CN/H_2O/CH_3COOH$ (49.5:49.5:1, v/v; pH 3.2) was used as a solvent instead.

Ligand Affinity Determination. *Initial Screening to Determine Approximate Binding Affinities.* First, the approximate binding affinities of thyroid hormones and tetrahalogen bisphenols to anti-T_4 Fab were determined via initial screening experiments (Figures S1−S7). Since the binding affinities were expected to be in the nanomolar range,[14,15] the protein concentration was fixed to 0.1 μM and each ligand was added at four to six different concentrations until the ligand saturation was observed (except in the case of TCB, for which the saturative binding was not observed even at a molar ligand excess of 200).

Direct Ligand Titration. A direct ligand titration[9,22,23] was used to determine the binding constant for the "low-affinity" reference ligand. Among the thyroid hormones tested, 3,3′-diiodo-L-thyronine (T_2) was selected as the reference ligand owing to its K_d value, fitting perfectly to a lowest practical protein concentration of 0.1 μM (see Results and Discussion section for details). Briefly, the concentration of anti-T_4 Fab was kept constant, while T_2 concentration was varied (0.005−2.0 μM), spanning approximately the K_d value estimated through initial screening experiments. The fractional saturation (Y) of the protein was calculated from the intensity ratio of the protein−ligand complex (PL) and free protein (P) for every single ligand (L) concentration (intensities averaged over different charge states), and the data were fitted into the specific, single-site binding model[24]

$$Y = \frac{B_{max}[L]}{K_d + [L]} \quad (1)$$

where B_{max} is the number of binding sites (maximum occupancy) and [L] is the free ligand concentration. Five replicate samples were prepared and measured for each T_2 concentration. The curve fittings were performed using OriginPro 15.0 software (Origin Lab, Northampton, MA, USA).

Ligand Competition Experiments. To determine the binding affinities for the other ligands, ligand competition experiments were performed.[25,26] Briefly, T_2 was used as the "low-affinity" reference ligand, whose accurate K_d value was determined earlier by the direct ligand titration. The two ligands, competing for the same binding site in anti-T_4 Fab, were mixed at the appropriate concentrations (determined earlier by initial screening experiments) with the protein at a fixed concentration of 0.1 μM. Ten replicates were measured for each ligand pair from three different samples. The final values have been reported as the mean value ± one standard deviation.

A mathematical model to calculate K_d values from the competition experiments was as follows: For L and L_{ref}, competing for a single binding site in P, the two equilibrium reactions are

$$P + L \leftrightarrows PL$$

$$P + L_{ref} \leftrightarrows PL_{ref}$$

where P is the receptor protein, L is the ligand, and L_{ref} is the "low-affinity" reference ligand. Briefly, the intensity ratio (R) of the total abundance of the ligand-bound protein (PL) and free protein (P) was calculated for each spectrum. The affinity measurement for the "low-affinity" ligand (L_{ref}) was performed

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by a direct titration method. The individual K_d values for L and L_{ref} are derived as

$$K_d(L) = \frac{[L][P]}{[PL]} \quad (2)$$

$$K_d(L_{ref}) = \frac{[L_{ref}][P]}{[PL_{ref}]} \quad (3)$$

where the brackets represent equilibrium concentrations of L, L_{ref}, P, PL, and PL_{ref}. The mass balance equations at equilibrium are

$$[P]_{total} = [P] + [PL] + [PL_{ref}] \quad (4)$$

$$[L]_{total} = [L] + [PL] \quad (5)$$

$$[L_{ref}]_{total} = [L_{ref}] + [PL_{ref}] \quad (6)$$

where $[P]_{total}$, $[L]_{total}$, and $[L_{ref}]_{total}$ are the total protein, ligand, and reference ligand concentrations, respectively.

At equilibrium, [P] is the same for both ligands, and thus, eqs. 4 and 5 were simplified to

$$K_d(L) = \frac{Kd(L_{ref})[PL_{ref}][L]}{[PL][L_{ref}]} \quad (7)$$

The ESI-MS spectrum directly provides normalized intensities ("concentration coefficients") i(P), i(PL), and $i(PL_{ref})$, such that their sum equals 1, which can then be used to calculate equilibrium concentrations by eqs 8, 9, and 10:

$$[P] = i(P)[P]_{total} \quad (8)$$

$$[PL] = i(PL)[P]_{total} \quad (9)$$

$$[PL_{ref}] = i(PL_{ref})[P]_{total} \quad (10)$$

These calculations are valid if one assumes that the ionization and ion transmission efficiencies for P, PL, and PL_{ref} are the same (they are if $m_L \approx m_{Lref} \ll m_P$, which is true in this case), and there is no complex dissociation upon ionization/desolvation stages (i.e., careful adjustments of instrumental parameters). The intensities should be calculated as integrated sums of different charge states to avoid any bias resulting from different CSDs between the free and bound proteins. The free ligand concentrations, [L] and $[L_{ref}]$, can be obtained from eqs 5 and 6. The equilibrium concentrations [L], $[L_{ref}]$, [PL], and $[PL_{ref}]$ as well as the predetermined/known $K_d(L_{ref})$ can be inserted into eq 7 to calculate K_d(L).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b02659.

Mass spectra of the initial ligand screening experiments (Figures S1−S7); mass spectra of the ligand competition experiment (Figure S8) (PDF)

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Notes

The authors declare no competing financial interest.

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