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Site-specific basicities regulate molecular recognition in receptor binding: in silico docking of thyroid hormones

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Abstract Interactions between thyroid hormone α and β receptors and the eight protonation microspecies of each of the main thyroid hormones (thyroxine, liothyronine, and reverse liothyronine) were investigated and quantitated by molecular modeling. Flexible docking of the various protonation forms of thyroid hormones and high-affinity thyromimetics to the two thyroid receptors was carried out. In this method the role of the ionization state of each basic site could be studied in the composite process of molecular recognition. Our results quantitate at the molecular level how the ionization state and the charge distribution influence the protein binding. The anionic form of the carboxyl group (i.e., carboxylate site) is essential for protein binding, whereas the protonated form of amino group worsens the binding. The protonation state of the phenolate plays a less important role in the receptor affinity; its protonation, however, alters the electron density and the concomitant stacking propensity of the aromatic rings, resulting in a different binding score. The combined results of docking and microspeciation studies show that microspecies with the highest concentration at the pH of blood are not the strongest binding ones. The calculated binding free energy values can be well interpreted in terms of the interactions between the actual sites of the microspecies and the receptor amino acids. Our docking results were validated and compared with biological data from the literature. Since the thyroid hormone receptors influence several physiologic functions, such as metabolic rate, cholesterol and triglyceride levels, and heart frequency, our binding results provide a molecular basis for drug design and development in related therapeutic indications.

Keywords Thyroid · Thyroxine · Receptor binding · Protein binding · Microspeciation · Molecular modeling

Introduction

Thyroid hormones are fundamental molecules regulating mammalian development and metabolism, including the development of the central nervous system in infants, skeletal growth in children, and also for normal function of multiple organ systems in adults (Stathatos 2012; Cody 1980). Thyroxine (T4), and to a lesser extent liothyronine (T3) and reversed liothyronine (rT3), are formed in the human thyroid gland by iodination and coupling reaction of tyrosine via monoiodotyrosine and/or diiodotyrosine (de Vijlder and den Hartog 1998). The constitutional formulas of thyroid hormones are shown in Fig. 1. T3 and rT3 are primarily produced in peripheral metabolism when T4 is converted into T3 or rT3 by deiodinase enzymes (Bianco et al. 2002). Thyroid hormones in the blood are bound to transport proteins. The main transport proteins are thyroxine-binding globulin (TBG), transthyretin (TTR), and human serum albumin (HSA), of which TBG has the

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highest affinity for thyroid hormones; it binds 75 % of serum T4, whereas TTR and HSA bind 20 and 5 %, respectively (Bartalena 1990). T4 is bound more tightly than T3 to each of the above proteins.

In spite of their high lipophilicity, thyroid hormones cross the cell membrane by energy-dependent, carrier-mediated transport (Hennemann et al. 2001). After membrane transport, T3 binds to the thyroid hormone receptors (TRs, a type of nuclear receptors) and modulates the transcription of specific genes. These receptors occur in two major subtypes: TRα and TRβ (Lazar 1993). TRα is expressed at higher levels in the heart, while TRβ predominates in the liver. Activation of TRα mainly affects the heart rate, whereas activation of TRβ is involved in hepatic cholesterol metabolism and regulation of metabolic rate (Forrest and Vennstrom 2000). Moreover, TRβ (subtype 2) has a key role in the feedback regulation of the hypothalamic–pituitary–thyroid (H–P–T) axis (Shibusawa et al. 2003). The binding pocket of TRs is conserved between TRα and TRβ with the exception of serine (Ser227) in TRα, which is substituted by asparagine (Asn331) in TRβ. This difference provides a chance to develop selective β receptor ligands (Dow et al. 2003; Wagner et al. 2001). There are intensive efforts to design TRβ-selective new entities and/or high-affinity thyromimetics (Malm et al. 2007; Du et al. 2008; Hangeland et al. 2004; Ye et al. 2003). A few molecules are known as selective TRβ agonists (GC-1, GC-24, KB-141) and are depicted in Fig. 1. Some derivatives of these compounds or their analogs have also been synthesized as potent TRβ-selective thyromimetics, such as a 6-azauracil derivative (Dow et al. 2003), a 4′-amido bioisosteric derivative of KB-141 (Li et al. 2006), 3-hydroxy-cyclobut-3-ene-1,2-dione derivatives (Raval et al. 2008), phosphonic acid derivatives (Boyer et al. 2008), etc. Structure–activity relationships of thyroid hormones have also been investigated (Andrea et al. 1979; Du et al. 2008). Thyroid hormone stereochemical properties play an important role in hormone activity. Only L-T4 has hormone effect, whereas the D-isomer has cholesterol-lowering activity. Disubstitution at 3- and 5-positions is essential for the effect that evolves the appropriate conformation. The 3′-monosubstituted compounds are more active than the 3′,5′-disubstituted ones. Truncation of the amino acid chain to an acetic acid moiety improves the effect. The 4′ phenolic –OH is necessary for receptor binding (Du et al. 2008).

Computational docking of a small molecule to a biological target involves efficient sampling of possible poses of ligands in the binding pockets of receptors to identify the optimal binding geometry and the crucial interactions between ligands and proteins (Mohan et al. 2005). However, molecular docking is a commonly used technique in drug design, with the following challenges: finding the optimal ligand–protein conformation in a natural/aqueous environment, predicting accurately the binding free energy, running the docking and scoring calculations in a reasonable time frame, etc. (Rester 2006).

Protonation states have long been recognized to play an important role in receptor binding. Nevertheless, only a few papers are thorough enough to take into account the different protonation states in protein–ligand docking calculations (Park et al. 2011). Furthermore, it is common practice for a single protonation form to be assigned to both the ligand and the receptor prior to the calculations, even though the identity of the binding protonation microspecies is usually not at all certain, especially if the ligand contains

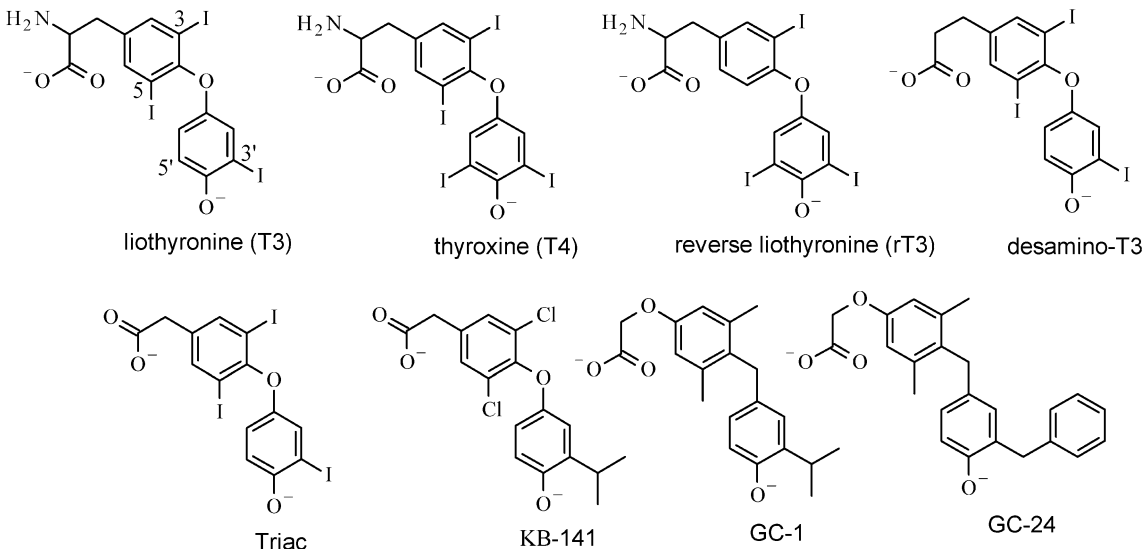

Fig. 1 Constitutional formulas of the thyroid hormones and high-affinity thyromimetics; all compounds are shown in their most basic form

more protonation centers of comparable basicity. On the other hand, amino acid residues in proteins surrounding the ligand are disposed to influence the local dielectric properties and pH. The ultimate docking strategy would therefore be if all the protonation and tautomer states could be docked to the receptor (ten Brink and Exner 2010). Furthermore, docking of more protonation microspecies to the receptors can deepen understanding of the ligand recognition process.

Natesan et al. (2011) investigated the role of both ionization and conformation in the binding affinities between thyroxine analogs and transthyretin, the second most important thyroid-transporter protein, using multispecies multimode comparative molecular field analysis (MS-MM CoMFA). Their results also demonstrate that the MS-MM modification, where more ionization species are investigated, improves the predictive abilities of the in silico method.

The pH-dependent affinity of thyroid hormones with solubilized nuclear receptors has been investigated (Latham et al. 1976; Wilson and Gent 1985). The authors report that the pH-dependent affinity profile of T3 shows a large increase of binding to nuclear receptor above pH 6 and the optimal pH range is between pH 7.1 and 7.9 (Wilson and Gent 1985). These data provide experimental evidence of the importance of the protonation states of ligands and/or proteins in molecular recognition.

We recently determined all the protonation macro- and microconstants of the main thyroid hormones (Toth et al. 2012, 2013). These data were in agreement with, and provided a rationale for, the biosynthetic yield, and receptor and transport protein binding ratios among T4, T3, and rT3, indicating that the protonation states of the thyroid hormones and their precursors are crucial properties to understand their biological functions and to influence the therapeutic properties of their derivatives in drug development efforts. In the present work, our aim is to investigate the interactions between protonation microspecies of thyroid hormones and receptor proteins using in silico modeling.

Here we report for the first time how the various microspecies of the three major thyroid hormones interact with the appropriate receptors, including the structural arrangements of the binding modes and quantitation of the binding strengths.

Methods

Individual protonation microspecies are highly elusive chemical entities. They occur only in continuous interconversion with nanosecond average individual lifetime, which makes them practically coexisting and inseparable by any currently known separation technique. However, they act in their individual form and have their own physicochemical and biochemical properties (Noszal 1990; Mazák et al. 2012). Docking studies are one of the best approaches to investigate the relationships between receptors and ligands. Using this method, the interaction between individual entities (with defined protonation, conformational, and rotational states) and proteins can be studied (Polgar et al. 2007).

We constructed each of the protonation microspecies and docked them flexibly to both TR isoforms. In this method the binding free energies and the possible interactions (hydrophobic, polar, and charge) between the amino acid residues of TR and thyroid hormones can be investigated in one step.

The ligand–protein cocrystal structures for the thyroid hormones and high-affinity thyromimetics with both receptor isoforms are available in the Brookhaven Protein Data Bank (http://www.pdb.org).

The crystal structures used in our docking studies (see "Docking to thyroid hormone receptors" section) and also in method validation (see "Method validation" section) are summarized in Table 1. The X-ray crystallography resolution of the used protein was less than 2.8 Å in all cases.

The protein model structures were prepared for docking and analysis using the Protein Preparation Wizard (Schrödinger Suite 2011) in the Schrödinger software graphical user interface Maestro (version 9.2). Hydrogens were added, reflecting the likely protonation states of amino acids at pH 7.4. Thus, the Arg and Lys side-chains were cationic, while the Glu and Asp side-chains were anionic. The lone waters and any other excess ligands were removed. For protein geometry optimization, the

Table 1 Crystal structures used in our docking studies

TR isoform	Ligand	PDB-ID	TR isoform	Ligand	PDB-ID
α	T3	2H77 (Nascimento et al. 2006)	β	T3	3GWS (Nascimento et al. 2006)
α	GC-1	3HZF (Bleicher et al. 2008)	β	GC-1	3IMY (Bleicher et al. 2008)
α	TRIAC	3JZB (Martinez et al. 2009)	β	TRIAC	3JZC (Martinez et al. 2009)
α	KB-141	1NAV (Ye et al. 2003)	β	KB-141	1NAX (Ye et al. 2003)
			β	GC-24	1Q4X (Borngraeber et al. 2003)

Table 2 Experimental and calculated binding free energies (in kcal/mol)

Ligand	ΔG_{exp}		ΔG_{calc}	
	TRα	TRβ	TRα	TRβ
T3	−12.9 (Boyer et al. 2008)	−12.6 (Erion et al. 2007)	−12.11	−12.67
GC-24	−10.73 (de Araujo et al. 2010)	−13.54 (de Araujo et al. 2010)	−10.96	−13.35
GC-1	−12.28 (de Araujo et al. 2010)	−13.70 (de Araujo et al. 2010)	−12.46	−13.29
TRIAC	−13.86 (de Araujo et al. 2010)	−14.04 (de Araujo et al. 2010)	−12.74	−11.99
KB-141	−11.08 (Erion et al. 2007)	−12.86 (Erion et al. 2007)	−10.87	−12.88
Desamino-T3	−15.04 (de Araujo et al. 2010)	−15.22 (de Araujo et al. 2010)	−14.52	−14.59

OPLS2005 force field was used (Jorgensen and Tirado-Rives 1988; Kaminski et al. 2001). This minimization process was terminated when the root-mean-square deviation of the heavy atoms in the energy-minimized structure relative to the starting, experimentally (X-ray) determined coordinates exceeded 0.3 Å (Sastry et al. 2013). For generation of the docking grid, the van der Waals radii of the nonpolar protein atoms were not scaled. The center of the grid box was defined by the ligand described in the PDB entry. For geometry optimization of ligands, the LigPrep Schrödinger module with the MMFF94s force field was used (Halgren 1999). These compounds were docked to the proteins using Glide (version 5.7) in extra precision (XP) mode (Glide 2011; Friesner et al. 2004). Free rotation of side-chain containing –OH or –SH groups was allowed in the receptor pocket, which can increase the accuracy of docking. The best three poses for each protonation microspecies of the compounds as evaluated by the Glide scoring function were taken (Friesner et al. 2006).

Results and discussion

Method validation

To prove the applicability of our method, we followed the validation procedure of de Araujo et al. (2010) with some modifications. Six molecules (T3, GC-24, GC-1, TRIAC, KB-141, and desamino-T3) (Fig. 1) with known dissociation constants (K_i values) for TRα and TRβ were docked to the receptor proteins. The ligands were extracted from the PDB files and docked to the protein. Table 2 lists the calculated and experimental binding free energies for each ligand investigated. Scatter plots of the experimental and calculated binding free energies are illustrated in Fig. 2.

Figure 2 shows that the correlation factor between ΔG_{exp} and ΔG_{calc} is appropriate ($r^2 = 0.7765$). The average difference between ΔG_{calc} and ΔG_{exp} is only 0.55 kcal/mol. The largest difference between the ΔG_{calc} and ΔG_{exp} values is observed for TRIAC, which is also in agreement with other molecular modeling works (de Araujo et al. 2010). The possible reason could be the stronger relationship between the carboxylate group and the iodine-containing aromatic rings, since the distance between these two moieties is shortest in TRIAC, and the concomitant, unusual electron distribution in this molecule.

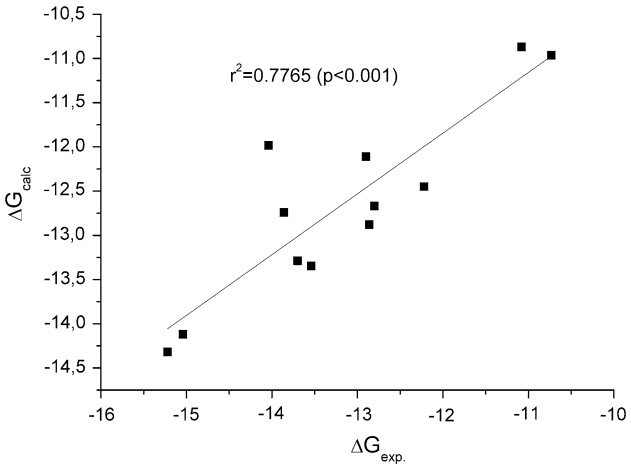

Fig. 2 Calculated versus experimental binding free energies for ligands in Table 2 to thyroid α and β isoforms; all values in kcal/mol

With this validation we proved that our docking method is appropriate to investigate the interaction between thyroid hormone microspecies and receptor proteins.

Acid–base equilibria

All the parent compounds studied are triprotic, with phenolate, amino, and carboxylate protonation sites. For a triprotic molecule, the total numbers of microspecies and microconstants are 8 and 12, respectively, as shown in Fig. 3.

The protonation scheme shows that, among the eight microspecies, there is one single chargeless one; all others bear at least one charged site. Each microspecies differs in charge and/or charge location, so their interactions with the receptor proteins are necessarily different.

In our previous papers (Toth et al. 2012, 2013) all the protonation macro- and microconstants were reported.

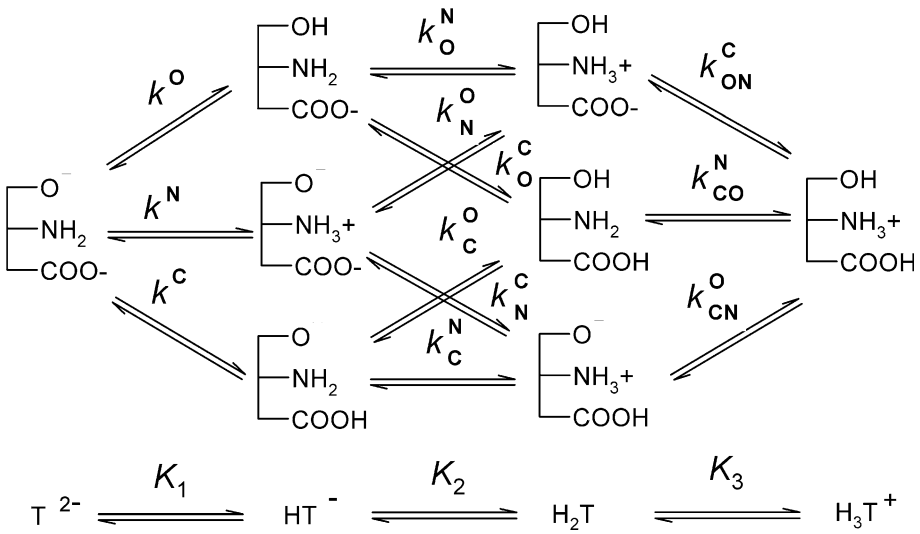

Fig. 3 Micro- and macrospeciation scheme of thyroid hormones, where microconstants with superscript "O", "N", and "C" correspond to the phenolate, amino, and carboxylate site, respectively. The superscript on the microconstant indicates the protonating site, while the subscript (if any) indicates the site already protonated. K_1, K_2, and K_3 are stepwise macroconstants

These values are summarized in Table S1 of the Electronic Supplementary Material. The pH-dependent distribution diagrams of the eight microspecies of the three compounds studied could be constructed on the basis of our determined protonation microconstants (Fig. 4a–c). Due to the different numbers and patterns of iodine substitution, the values of the protonation microconstants, especially those of the phenolate, are different in the investigated hormones. Our pH-dependent distribution diagrams show that T4 and rT3 mainly exist at blood pH in the (O^-, NH_3^+, COO^-) form, whereas T3, the biologically most active hormone, occurs predominantly in the (OH, NH_3^+, COO^-) state. Furthermore, in T3 the concentration of the two anionic microspecies (OH, NH_2, COO^-) and (O^-, NH_3^+, COO^-) is comparable because of the similar basicity of the amino and phenolate groups. These differences may also contribute to the different binding properties of the thyroid hormones and warn that docking only one protonation species to the receptors is insufficient to map the correct structure–activity relationship, or to understand the multifactorial interactivity between the ligand and receptor.

Docking to the thyroid hormone receptors

All 24 protonation microspecies were docked to the wild-type TRs. The calculated binding free energies are summarized in Table 3.

The values in Table 3 clearly show that microspecies of T3 exhibit the best binding to both receptor isoforms, while the binding of rT3 is the poorest. This is in good agreement with the physiological affinity results (Andrea et al. 1979; Farwell and Braerman 2006). T4 is actually the precursor of T3, the latter being, in fact, the biologically active hormone, while rT3 is ineffective. This can be explained based on the observation that disubstitution in the outer thyronine ring precludes formation of a hydrogen bond between the histidine and the 4′ hydroxyl moiety of the ligand as shown in Fig. 5a, b.

It is known that disubstitution in the inner thyronine ring is required to achieve the correct binding conformation. The poorest binding free energy values of rT3 are well interpretable in light of its doubly hampered binding propensity: this molecule is disubstituted in the outer thyronine ring and monosubstituted in the inner thyronine ring.

Our binding study can also explain the kinetics of thyroid hormones. Thyroid hormones associate fast with a monomer form of TRs (K_{on} = 0.02–0.14 h); it can be seen, however, that T4 dissociates from TRs more rapidly than T3 due to the bulky 5′ iodine atom. Consequently, the TR–T4 complexes [$\frac{K_{on}}{K_{off}}$ (T4–TRα) = 0.53, $\frac{K_{on}}{K_{off}}$ (T4–TRβ) = 1.33] are less stable than the TR–T3 complexes [$\frac{K_{on}}{K_{off}}$ (T3–TRα) = 0.02 h, $\frac{K_{on}}{K_{off}}$ (T3–TRβ) = 0.01 h] (Sandler et al. 2004; Cunha Lima et al. 2009; Cunha Lima and Rodrigues 2011).

The calculated binding free energies in Table 3 also show that the protonation states of the basic sites strongly influence the binding strength and mode, as follows: The most influential factor is the protonation state of the amino acid side-chain. Microspecies in which the amino and carboxylate groups are in deprotonated form have the best docking, while the microspecies in which the amino and carboxylate are in protonated form have the poorest. The receptor–ligand interactions between the two microspecies (NH_2, COOH, O^-) and (NH_3^+, COOH, OH) with TRβ are presented in Fig. 6a, b.

It can be observed that the binding pockets of TRα and TRβ contain several aromatic amino acid side-chains which can undergo hydrophobic interaction with the iodine-containing aromatic rings. In addition, there are two polar regions in the binding pockets: One of them is a

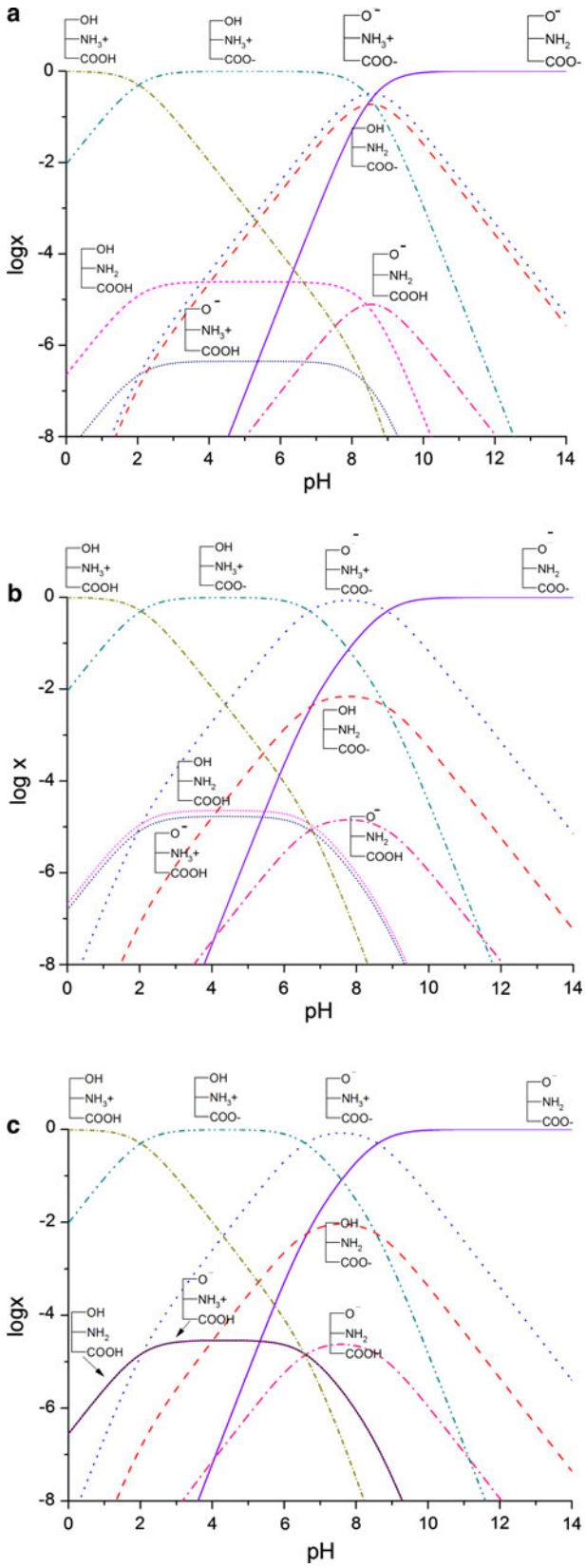

Fig. 4 Logarithmic distribution diagrams for all the microspecies of T3 (**a**), rT3 (**b**), and T4 (**c**)

single histidine which forms a hydrogen bond with the phenolic hydroxyl of the outer thyronine ring. The most polar interactions are formed between arginine residues and Ser227 in TRα and Asn331 in TRβ and the amino acid side-chain of T3. Protonation is unfavorable for these latter important interactions. Protonation of the amino group generates a cationic site and a concomitant repulsive electrostatic interaction with the positively charged arginine guanidinium residues. The orientation of the flexible amino acid side-chains will consequently be turned, and the possibility for the strongest secondary interaction will be cancelled, so the receptor cannot accommodate the ligand in the appropriate pocket location. In all cases the binding free energies of the microspecies with protonated amino and/or carboxylate group(s) are less favorable (smaller negative numbers) than the binding free energies of the microspecies with nonprotonated functional group(s). It is also remarkable that desamino-T3 (Fig. 1), the T3 derivative without amino group, has a better docking score than the parent molecule. This is in agreement with the biochemical fact that the amino group reduces the receptor affinity; however, it plays an important role in hormone transport and in metabolism (Farwell and Braerman 2006).

The protonation state of the phenolate exerts only a minor influence on the binding, because the hydrogen bond between the histidine imidazole and the phenolate can be formed in either the –OH or $–O^-$ phenol(ate) form. Nevertheless, the phenolate protonation state is in canonical conjugation with the electron density of the aromatic rings, which slightly influences the stacking interactions and the binding free energies.

T3 occurs in 86 % as (OH, NH_3^+, COO^-) at the pH of blood (Toth et al. 2012). The binding free energies show, however, that this is not the best binding microspecies, which is in fact (NH_2, OH, COO^-). The presence of this T3 microspecies is only 5 % at pH 7.4 (Fig. 4a). Comparing, however, the presence of the (NH_2, OH, COO^-) form in T4, T3, and rT3, it is highest in T3, which clearly explains the ranking of the thyroid hormones in terms of their biological activity. This study also exemplifies that not always is the major microspecies the best receptor-binding one, which may rather have good pharmacokinetic properties. In the process of receptor docking, the ligand can only bind the receptor in a specified form (in definite protonation and conformational states). Therefore, knowledge of the concentration of each microspecies at different pH values can be crucial to understand the behavior of drugs and biomolecules at the molecular level. For docking studies, an important consequence to highlight is that not only the major species need to be docked to the receptor, since the protonation state greatly influences the docking score.

Table 3 Calculated binding free energies of the protonation microspecies of the three thyroid hormones (in kcal/mol)

	T3 (TRα)	T3 (TRβ)	T4 (TRα)	T4 (TRβ)	rT3 (TRα)	rT3 (TRβ)
NH_2, COO^-, O^-	−12.11	−12.69	−11.15	−10.44	−7.05	−7.62
NH_3^+, COO^-, O^-	−9.46	−10.65	−8.69	−9.29	−5.17	−6.41
NH_2, COOH, O^-	−9.30	−8.47	−8.45	−9.34	−5.87	−6.01
NH_3^+, COOH, O^-	−4.83	−6.35	−4.31	−6.80	−3.89	−5.74
NH_3^+, COO^-, OH	−11.70	−11.70	−10.40	−9.71	−8.44	−8.58
NH_2, COO^-, OH	−13.18	−11.75	−12.15	−11.07	−9.51	−9.73
NH_2, COOH, OH	−9.96	−10.13	−9.53	−9.76	−7.13	−8.21
NH_3^+, COOH, OH	−5.30	−6.86	−5.75	−6.98	−5.12	−6.49

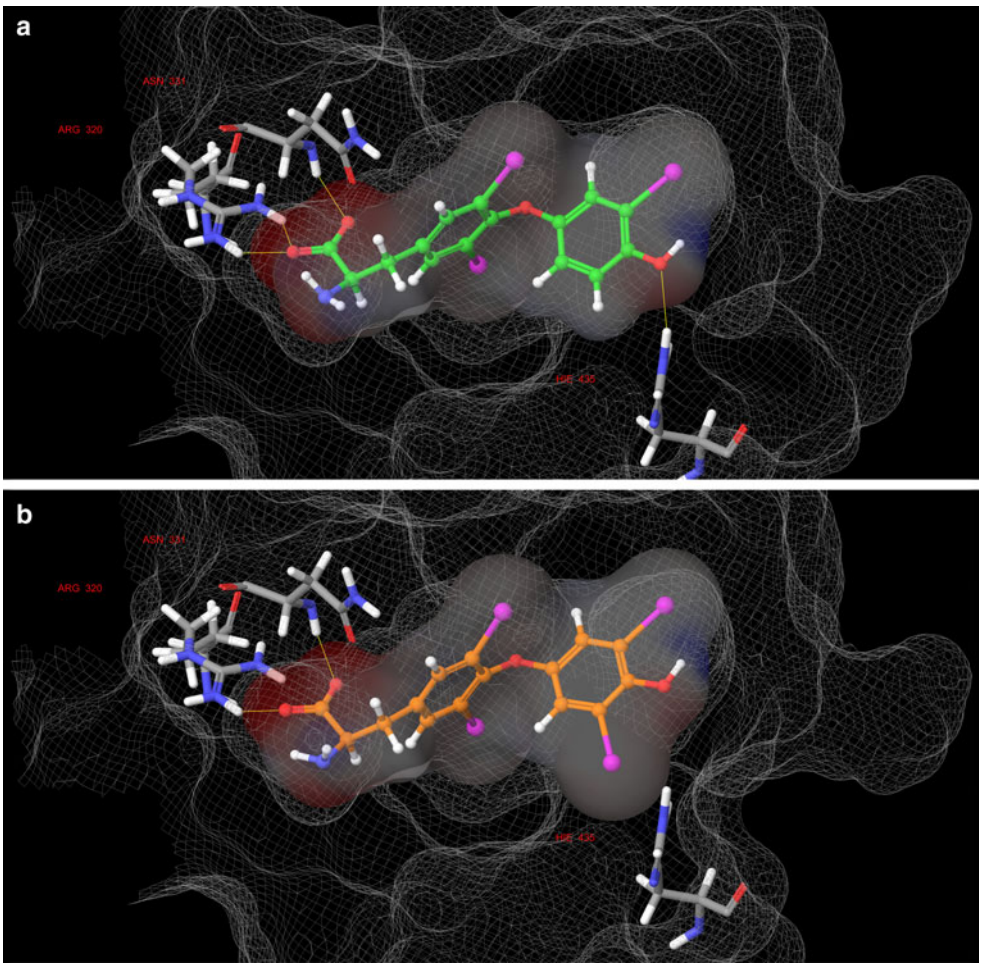

Fig. 5 **a** Interactivity between T3 and TRβ. **b** Interactivity between T4 and TRβ. In T4 the iodine atoms at 3′ and 5′ positions block the formation of a hydrogen bond between histidine (HIE435) and 4′ hydroxyl group of the ligand

We also investigated how the binding conformation changes with the protonation state. The superposition of all the T3 docked microspecies is depicted in Fig. 7. In accordance with data and statements above, the docked position of the phenol group changes much less than the amino group. The average difference between the phenolate oxygen of the X-ray structure and phenolate oxygen of the docked microspecies is 0.67 Å, and the largest difference is only 1.4 Å. Contrary to that, the average difference between the amino nitrogen of the X-ray structure and the amino nitrogen of the docked microspecies is 1.83 Å, and the largest difference is 3.3 Å. It can also be seen that, upon protonation of the amino group, the fit of the receptor pocket deteriorates.

Conclusions

Thyroxine, liothyronine, and reverse liothyronine microforms with different protonation patterns, constituting a

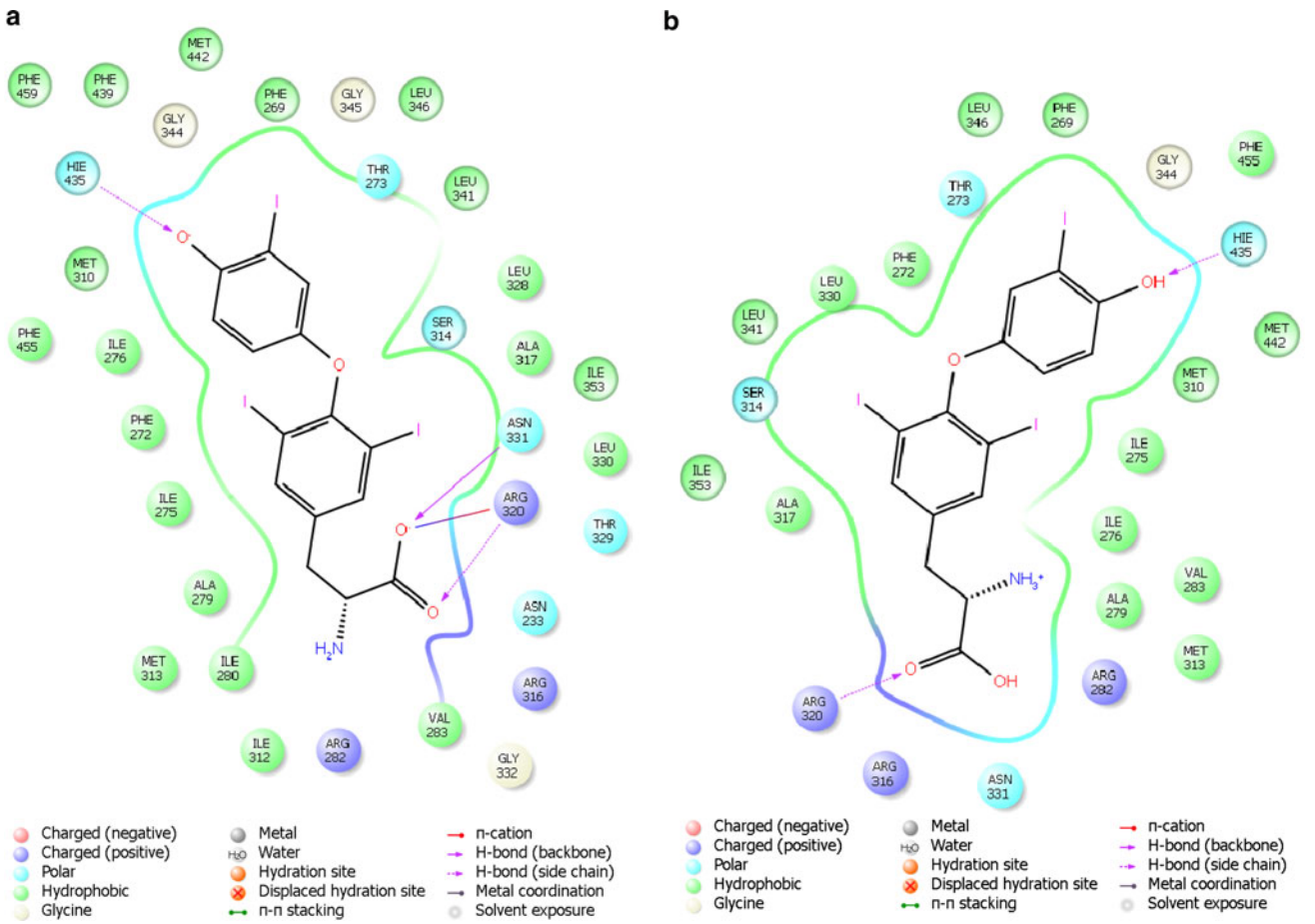

Fig. 6 Receptor–ligand interactions between TRβ and the T3 microspecies **a** (NH_2, COOH, O^-) and **b** (NH_3^+, COOH, OH)

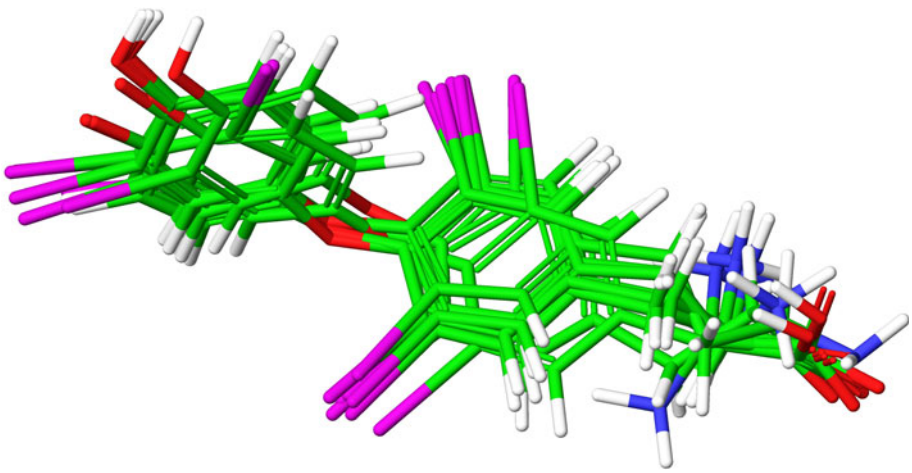

Fig. 7 Superposition of the docked T3 microspecies. The docked position of the phenol group changes much less than that of the amino group

total of 24 microspecies, were docked to thyroid hormone α and β receptors, and the resulting 48 docking scores were compared with those of five high-affinity thyromimetics. The major conclusions of the results are as follows:

Strong binding occurs when both the amino and carboxylate sites are in their basic (deprotonated) form. Protonation of either of these groups diminishes the binding propensity. The protonation state of the phenolic –OH influences the binding strength only slightly, since hydrogen bonding can be formed in both proton-donor and proton-acceptor fashions. The best binding microspecies are not those that occur in highest concentration at the pH of blood.

Our study reveals the intermoiety interaction between the three main thyroid hormones and their receptors at the

molecular level, which provides the fundamentals for the design of selective drugs in several thyroid-related disorders.

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