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Review

The thermodynamic and binding properties of the transferrins as studied by isothermal titration calorimetry☆

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ABSTRACT

Background: In mammals, serum-transferrins transport iron from the neutral environment of the blood to the cytoplasm by receptor-mediated endocytosis. Extensive *in-vitro* studies have focused on the thermodynamics and kinetics of Fe^{3+} binding to a number of transferrins. However, little attention has been given to the thermodynamic characterization of the interaction of transferrin with its receptor.
Scope of review: Iron-loaded transferrin (Tf) binds with high affinity to the specific transferrin receptor (TfR) on the cell surface. The Tf–TfR complex is then internalized via receptor mediated endocytosis into an endosome where iron is released. Here, we provide an overview of recent studies that have used ITC to quantify the interaction of various metal ions with transferrin and highlight our current understanding of the thermodynamics of the transferrin–transferrin receptor system at physiological pH.
General significance: The interaction of the iron-loaded transferrin with the transferrin receptor is a key cellular process that occurs during the normal course of iron metabolism. Understanding the thermodynamics of this interaction is important for iron homeostasis since the physiological requirement of iron must be appropriately maintained to avoid iron-related diseases.
Major conclusions: The thermodynamic data revealed stoichiometric binding of all tested metal ions to transferrin with very high affinities ranging between 10^{17} and 10^{22} M^{-1}. Iron-loaded transferrin (monoferric or diferric) is shown to bind avidly ($K \sim 10^{7}$–10^{8} M^{-1}) to the receptor at neutral pH with a stoichiometry of one Tf molecule per TfR monomer. Significantly, both the N- and the C-lobe contribute to the binding interaction which is shown to be both enthalpically and entropically driven. This article is part of a Special Issue entitled Transferrins: Molecular mechanisms of iron transport and disorders.

1. Introduction

During the past two decades, a large number of studies were directed towards our understanding of the biochemistry and biology of transferrin (Tf) and the transferrin receptor (TfR), including structure, metal-binding, functional and therapeutic properties, regulation of cellular iron homeostasis and the mechanism of TfR-mediated iron uptake by cells and transport across the blood–brain barrier [1–4]. The serum transferrin system involves the specific recognition of ferric Fe^{3+} ions alongside a synergistic anion (usually carbonate). At physiological pH, TfR has a high binding affinity for iron-loaded Tf whereby iron is released inside the cell following receptor-mediated endocytosis. Upon release of iron, the Tf–TfR complex returns to the cell surface where the apo-transferrin dissociates from the receptor at physiological pH and is released into the circulation for another cycle. The half-life of transferrin in the human circulation is 7.6 days, and the lifetime of transferrin-bound Fe is 1.7 h. The entire cycle of endocytosis is complete within a few minutes and is estimated to occur 100–200 times during the lifetime of the transferrin molecule [5–7].

2. The transferrins: a brief historical background—structure and properties

The isolation and purification of the first member of the transferrin superfamily from raw egg white, ovotransferrin, was performed by Osborne and Campbell in 1900 [8]. Almost half a century later, iron-binding proteins from human serum, serum transferrin, and from human milk, lactoferrin, were isolated by a number of laboratories [9–12]. Another member of the transferrin superfamily is melanotransferrin [13], a cell-surface glycoprotein found in melanocytes and a number of other tissues. Melanotransferrin (MTf) does not appear to play a role in iron metabolism but has an important role in melanoma cell proliferation and tumorigenesis [14] and is discussed in depth elsewhere (see Richardson's chapter in this special issue). There is about 60% sequence identity between serum transferrin and lactoferrin and about 40% identity between MTf and other

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transferrins [2]. Transferrin was also found in a number of other body fluids such as tears and saliva. Their potent anti-inflammatory and bacterial activities is mainly due to their extremely high iron binding affinity which limits the amount of iron available for microbial growth [15–21]. The reader is urged to refer to Rascon–Cruz's chapter in this special issue for additional information on lactoferrin and its functions.

Modern transferrins are believed to have evolved by gene fusion and duplication from an ancestral monolobal transferrin with a single metal-binding pocket [22,23]. Two monolobal transferrins were first isolated from the ascidian *Pyura stolonifera* [24] and *Halocynthia roretzi* [25]. More recently, monolobal transferrin from the plasma of the ascidian *Ciona intestinalis*, named nicatransferrin (nicaTf) was isolated and characterized for its metal-binding capacity [26]. NicaTf was found to have a high sequence homology to the N-lobe of modern bilobal transferrins with conserved Fe(III)-binding residues. However, the significance of this finding in the evolution of the modern family of bilobal transferrin remains unclear [27]. Modern transferrins are a family of homologous monomeric bilobal glycoproteins of molecular weight approximately 80 kDa with a crucial role in iron homeostasis. They are composed of a single polypeptide chain of approximately 700 amino acid residues and bind tightly and reversibly two Fe(III) ions with the assistance of carbonate (or bicarbonate), the physiological anion, although other synergistic anions have been shown to concomitantly bind [2 and references therein]. The synergistic carbonate anion provides two oxygen ligands needed to complete the octahedral coordination sphere of the binding site and might play a role in iron release through protonation and disruption of the hydrogen bonding network around the metal ion. Alternatively, the bridging carbonate anion firmly locks the metal ion in the binding pocket of the protein thus preventing water from binding and avoiding hydrolysis [28]. The two iron-binding pockets are about 42 Å apart and comprise four highly conserved residues, two tyrosines, one aspartate, one histidine and an arginine residue involved in the anchoring of the carbonate anion to the protein [2,28–31].

While transferrin is primarily an iron-binding and transport protein with a very high affinity for iron (in the range of 10^{21}–10^{22} M^{-1}), it is only about 30% saturated with iron in normal human serum. A number of metal ions (up to 40 different metal ions) have been shown to bind to transferrin [3,4,32–38] in the presence of different synergistic anions such as bicarbonate, oxalate or nitrilotriacetate and include Mn(II), Cu(II), Ni(II), Cd(II), VO(II), Zn(II), Cr(III), Co(III), Ru(III), Bi(III), Ga(III), In(III), Al(III), Tl(III), La(III), Ce(III), UO_2(II), Nd(III), Sm(III) and Gd(III). While some of these metal ions are toxic, with perhaps no physiological significance or benefits, such binding may play an important role in the transport and delivery of metal-containing compounds for diagnosis and therapy particularly that hetero-metal transferrin complexes are still recognized by the transferrin receptor. For example, platinum complexes are currently being used in cancer chemotherapy, gold compounds are employed in the treatment of arthritis, gallium, indium and aluminum are used as medical diagnostic radioisotopes, and bismuth, ruthenium and titanium are used as anti-ulcer medication [2,39 and references therein].

2.1. The transferrin receptors—TfRs

Two transferrin receptors have been described, TfR1 and TfR2 with TfR1 being the more extensively characterized. Both of them are homodimeric type II cell transmembrane glycoprotein receptors that only bind iron-loaded transferrin whereby the diferric form exhibits higher affinity to the receptor than either monoferric forms (C-loaded or N-loaded transferrin) at neutral pH [2,28]. TfR1 is a disulfide bonded dimer consisting of two identical monomers with molecular mass ~95 kDa each. The large extracellular ectodomain portion has a molecular mass of approximately 70 kDa and contains the transferrin molecule binding site. The two receptors share about 45% amino acid sequence identity and about 65% similarities in their extracellular domains. The affinity of TfR1 for diferric transferrin is considerably higher than that of TfR2 with the dissociation constants for the two receptors, in their soluble forms being approximately 1 nM and 30 nM for TfR1 and TfR2, respectively [40]. Knock-out studies of TfR2 in mice showed that TfR2 is required for normal systemic homeostasis and does not compensate for TfR1 knock-out mutation [41–44]. A more detailed description of the transferrin superfamily members, their associated receptors and interacting partners and the regulation of iron homeostasis by transferrin receptor 2 (TfR2) is provided elsewhere in this volume (see the chapters by “Chen and Enns”, “Lambert”, and “Geiser and Winzerling”).

Indirect methods, such as ELISA and Surface Plasmon Resonance (SPR) have been routinely used in the characterization of molecular interactions. Other methods (e.g. dialysis, ultracentrifugation) rely on the direct determination of the different species present in the system (free macromolecule, free ligand and macromolecule/ligand complex) while spectroscopic and calorimetric techniques measure a signal that is proportional to the advance of the reaction (formation of macromolecule/ligand complex). When using spectroscopic techniques several experiments at different temperatures must be performed in order to estimate the binding enthalpy from the temperature dependence of the association constant (e.g. Van't Hoff equation). Isothermal titration calorimetry (ITC) has evolved as a valuable tool to characterize binding interactions and the molecular forces that drive them. It is thus considered the preferred technique in many fields of science due to a number of practical reasons such as accuracy, time required to run an experiment and amount of sample needed. Furthermore, ITC is a true in-solution method that requires no chemical tagging or immobilization of binding components and offers the advantage of working with either colored solutions, turbid or particulate suspensions. When combined with structural information, the elucidation of the nature and magnitude of the forces that are responsible for molecular interactions is an important step towards the understanding of the physical basis of macromolecular recognition and cellular mechanisms. This chapter provides an overview of our current understanding of the molecular interaction of Tf with TfR as characterized by microcalorimetry. In particular, it addresses the thermodynamics of binding of various metals to transferrin and the interaction of Tf and a number of mutants with TfR.

3. Isothermal titration calorimetry (ITC)

ITC is an emerging and powerful technique that is widely used to obtain in-solution biophysical data and evaluate the thermodynamic quantities involved in biochemical reactions or association equilibria. While the theoretical background and other practical aspects of ITC are discussed in detail elsewhere [45–51], a brief description of the technique and experimental design will be provided here. Notwithstanding limitations, ITC is considered the gold standard for measuring biomolecular interactions. Modern titration nano-calorimeters are able to accurately measure very small amounts of heat change (<0.2 μJ) in aqueous solution upon the delivery of a ligand. In a single experiment, ITC measures the association constant (K), stoichiometry (n) and the enthalpy change (ΔH^0) of binding. Because K is related to the Gibbs free energy of binding (ΔG^0), the entropy change of the reaction can thus be easily calculated from the relationship $\Delta G^0 = -RT\ln K = \Delta H^0 - T\Delta S^0$. Most of the commonly used isothermal titration calorimeters are based on a cell feedback network which measures the differential heat effects between a sample and a reference cell. This is known as differential power compensation and is due to a very small temperature difference between the two cells and is the baseline level in the absence of any reaction. When a reaction occurs, a temperature change is observed between the sample and the reference cell and is detected by the calorimeter. Thus, exothermic

reactions trigger a temporary decrease in the feedback power while endothermic reactions trigger an increased feedback. The heat evolved or absorbed by the reaction is then obtained by integration of these deflections from baseline, with respect to time.

In a typical ITC experiment, the macromolecule is placed in the sample cell while the reference cell contains buffer or water minus the macromolecule. The heat absorbed or evolved during a calorimetric titration is directly proportional to the fraction of bound ligand. For the initial injections, all or most of the added ligand is bound to the macromolecule, resulting in large endothermic or exothermic signals, depending on the nature of the association. As the ligand concentration increases, the macromolecule becomes saturated and subsequently less heat is evolved or absorbed on further addition of titrant. A binding curve is then obtained from a plot of the heats from each injection against the ratio of ligand to binding partner in the ITC reaction cell. Analysis of this binding curve using the appropriate binding model will lead to the determination of all thermodynamic parameters. It is important to note that the range of binding affinities that could be measured by modern ITC instruments is normally between 10^3 and $10^9\ M^{-1}$ and that the observed heat usually contains heat effects from several sources and side reactions and these include heat of binding (to be measured), heat of dilution of ligand, heat of mixing, heat of protein conformational changes upon ligand binding, heat of protonation or deprotonation of reactants or products, heat of metal-ion-buffer complexation, etc. Because it is not trivial to isolate the heat from these different sources, often the heat obtained by ITC is the net heat of reaction which includes contributions from all reactions involved in the binding process. Consequently, the ITC experimental conditions will dictate the extent of variability of thermodynamic quantities depending on the nature and effect of the side reactions. Nevertheless, control experiments in the absence of the macromolecule are typically performed to correct for some of the unwanted heat effects particularly those due to both dilution and mixing.

Despite the importance of ITC and its ease of use, the correlation between thermodynamic parameters and molecular structural features is somewhat under-valued. Nevertheless, a lot of attention has been diverted to ITC and its application to many diverse fields ranging from biological problems to optimization of lead compounds and pharmaceutical development and nanotechnology [52–61]. For example, dissection of binding affinity into the separate enthalpic and entropic contributions provides valuable information with regard to the binding mode of a ligand or how a potential drug interacts with the target, which can be used to build structure–activity relationships and computer-aided drug design. In terms of biological application, a number of ITC studies characterizing the thermodynamics of different metal ions binding to ferritin have appeared in recent years [52–55]. In a recent review [56], Wilcox highlighted a number of studies that employed ITC to look at various metal ions binding to proteins. The review provided new insights into the thermodynamics of metal–protein interactions beyond the simple determination of binding stoichiometry and affinity constants and showed the importance of quantifying enthalpic and entropic contributions in trying to understand the meaning of thermodynamic changes leading to metal–protein complex stability.

It is important to note that while the observed thermodynamic parameters (e.g. ΔG^0, ΔH^0 and ΔS^0) represent the change in state of the system, it is not always easy to understand or unambiguously interpret their meaning, particularly in the case of complex systems with multiple binding sites, cooperativity, etc. Nevertheless, it is of interest to briefly mention what these parameters generally describe. The Gibbs free energy change, ΔG^0, which is derived from the association constant K, indicates the binding affinity or strength of the interaction. The enthalpy of binding ΔH^0 reflects the interaction of the binding macromolecules relative to the individual macromolecules with the solvent. These contributions are primarily due to hydrogen bond formation, electrostatic and van der Waals interactions. The entropy of binding ΔS^0 is the harder to understand conceptually but it reflects a change in the degree of order of the system. In many cases, large and positive entropy values are often due to the changes in the hydration of the protein and of the ligands upon binding. Entropy changes are also influenced by changes in conformation of the macromolecules (e.g. folding/unfolding) and the restriction of degrees of freedom of the macromolecules main chain and side groups [56–61].

4. Thermodynamic characterization of iron binding to transferrin

4.1. Ovotransferrin and serum transferrin

Almost two decades ago, Brandts and coworkers employed sensitive isothermal titration calorimetry to investigate the binding of ferric ion to the N-site and C-site of ovotransferrin (oTf) and human serum transferrin (hTf) under various experimental conditions [62,63]. These include different protein concentrations, double- and single-domain transferrin (full length N-site and C-site molecules and only N-site and C-site terminal half-molecules), the presence or absence of the synergistic carbonate anion, and variations in pH, buffer and temperature. The proteins were titrated with an iron solution added in the form of an iron–nitrilotriacetic acid complex (Fe–NTA) where NTA was present in a two-fold molar excess. The two studies showed that the thermodynamics and kinetics of the binding events were quite different for the two sites of the two proteins. The data indicate that the two

Table 1
Titration calorimetry data for the binding of Fe-NTA to ovotransferrin (oTf) and human serum transferrin (hTf) in the presence (25 mM) and absence of bicarbonate in 100 mM Hepes, pH 7.5 and 27 °C. Endo and Exo refer to endothermic and exothermic reactions (With kind permission from Refs. [62,63]).

	oTf	hTf
Two thermodynamic events		
1st event	Binding to N-site/Endo $K_1/K_2 = 340$; $\Delta H^0 = 0.75$ kcal/mol	Binding to C-site/Exo $K_1/K_2 = 150$; $\Delta H^0 = -10.7$ kcal/mol
2nd event	Binding to C-site/Exo $K_1/K_2 = 340$; $\Delta H^0 = -12.1$ kcal/mol	Binding to N-site/Exo $K_1/K_2 = 150$; $\Delta H^0 = -4.2$ kcal/mol
Bicarbonate binding	Fast in the N-site/Endo Slower in the C-site/Exo	Fast in the C-site/Exo Slower in the N-site/Endo
Fe binding (in the presence of Bicarbonate)		
1st phase (Initial Fe-NTA contact)	Rapid/Exo for both sites	Rapid/Exo for C-site
2nd phase (Insertion of bicarbonate)	Slow/Endo for N-site; Exo for C-site	Fast/Exo for C-site; Slow/Endo for N-site
Fe binding (in the absence of Bicarbonate)		
	Rapid/Exo for N-site followed by C-site $K_{1(N\text{-site})} = 25\times10^6\ M^{-1}$; $\Delta H = -7.6$ kcal/mol $K_{2(C\text{-site})} = 2.3\times10^6\ M^{-1}$; $\Delta H = -3.6$ kcal/mol	Rapid/Exo for C-site followed by N-site $K_{1(C\text{-site})} = 3.3\times10^7\ M^{-1}$; $\Delta H = -8.7$ kcal/mol $K_{2(N\text{-site})} = 3.9\times10^6\ M^{-1}$; $\Delta H = -5.6$ kcal/mol

binding sites in these proteins, which are separated by some 40 Å, are not independent of one another but communicate as a result of ligand-dependent changes in the heats and free energies of domain–domain interactions. For example, the preferential binding of bicarbonate and iron to the N-site over the C-site of ovotransferrin is due to the faster rate of bicarbonate insertion and higher binding constant, respectively, while it is the exact opposite with serum transferrin. Table 1 summarizes the bicarbonate and iron-binding events in both proteins as observed by ITC.

4.2. Melanotransferrin

Melanotransferrin (MTf), also called p97, is a 97-kDa protein and a member of the transferrin superfamily. It shares about 38% sequence homology with human transferrin, human lactoferrin, and ovotransferrin [64,65]. However, unlike human transferrin, MTf is a membrane-bound transferrin and does not seem to efficiently donate iron to cells [13,66–69]. While the iron-binding ligands in the N-lobe of MTf and hTf are identical and able to bind Fe [70,71], several critical amino acid substitutions render the C-lobe of MTf incapable of binding iron [72]. A recent study employing MTf knockout mice showed no differences with wild type mice suggesting no essential role for MTf in iron metabolism [73]. An earlier study suggested the existence of another MTf gene which encodes a soluble form of the protein that can be found in the serum of patients with various disease states, including Alzheimer's disease [74]. However, the role of this soluble MTf protein is not clear but it is suspected to be implicated in the biological role of MTf [75].

The binding affinity of iron to MTf was studied by ITC and DSC (Differential Scanning Calorimetry) and was found to be similar to other known transferrins (i.e. $K_{ITC} = 2.6 \times 10^7\ M^{-1}$ and $\Delta H = -35.95$ kJ/mol in the absence of carbonate, 100 mM Hepes, pH 7.5, 25 °C (Table 2) and $K_{DSC} = 4.4 \times 10^{17}\ M^{-1}$, under the same conditions but in the presence of 25 mM bicarbonate) [76]. Consistent with the amino acid sequence alignment [72], both ITC and DSC data revealed only one iron-binding site in the N-lobe of the molecule [76]. The authors argue that MTf might provide another mechanism for iron delivery to cells in case Tf is absent from the medium. Their argument is based on the apparent high affinity constant of iron for MTf ($\sim 10^{17}\ M^{-1}$) and the fact that mammalian cells in culture may express $\sim 10^6$ molecules of MTf/cell [76,77]. Using mixed glial cultures from hypotransferrinemic mice [78], which lack Tf, the authors suggest that another dynamic metal exchange system (other than Tf–TfR) was responsible for the non-Tf-bound iron uptake into the tissues [78,79]. Interestingly, receptor-bound diferric transferrin releases its iron preferentially from the C-lobe at low pH [80] which probably explains why circulating transferrin has its N-lobe "always" loaded with iron. It was thus proposed that a major role of the N-lobe in transferrin is to facilitate iron release from the C-lobe [81,82]. However, in the case of MTf where iron is only found in the N-lobe, the protein might provide a back-up mechanism for iron delivery to cells in the absence of a Tf–TfR system. Interestingly, in an earlier study on the release of iron from the two iron-binding sites of transferrin by human erythroleukemia (K562) cells, the N-terminal site of transferrin was found to lose its iron before the C-terminal site at a higher pH induced by exposing the cells to increasing concentrations of the weak base methylamine [83].

5. Isothermal titration calorimetry studies of titanium and vanadium binding to hTf

As mentioned earlier, apo-human serum transferrin is known to bind a wide variety of metal ions. The strength of binding appears to be determined by the acidity of the metal ion [84–86] and the ligand donor [87] rather than the size of the ion. The kinetics of metal ions (i.e. aluminum, gallium, bismuth, uranyl and cobalt) uptake and release by transferrin upon interaction with its receptor is covered by El Hage Chahine in a separate chapter of this special issue. Nonetheless, recent studies employing isothermal titration calorimetry have looked into the binding of metal ions (other than iron), particularly titanium, Ti(IV) and vanadium species, to transferrin (more below). A more recent study investigated the binding of transferrin to ruthenium complexes which have been shown to be active against resistant malaria and several lines of cancer cells [88]. The ITC data showed several binding sites on the apo-protein with binding affinities in the range of 10^3 to $10^5\ M^{-1}$ depending on the type of Ru-complex used.

5.1. Titanium binding to hTf

In the presence of ≥3 equivalents of citrate, the $\{Ti(cit)_3\}^{3-}$ was found to be the predominant species in the pH range of 6 to 9 [89] and a stable complex that would rapidly deliver Ti(IV) to Tf [90]. As with the case of iron binding to Tf, the binding stoichiometry was found to be one Ti(IV) per site, with the N- and C-lobe exhibiting different binding affinities (i.e. one order of magnitude higher affinity to the C-site than the N-site) [90]. The ITC affinity constants and change in enthalpy in the absence of bicarbonate were determined to be $K_C = 3.2\ 10^6\ M^{-1}$, $\Delta H = -8.03$ kJ/mol and $K_N = 2.4 \times 10^5\ M^{-1}$, $\Delta H = -4.89$ kJ/mol for the C-lobe and the N-lobe, respectively (Table 2). When accounting for the stability constant of $\{Ti(cit)_3\}^{3-}$, the pH and the bicarbonate concentration, the thermodynamic stability constants for Ti(IV) binding to the two lobes of Tf were calculated to be log $K_C = 26.8$ for the C-lobe and log $K_N = 25.7$ for the N-lobe [90] suggesting

Table 2
Thermodynamic parameters of metal ions binding to transferrins, pH 7.4–7.5 and 25–27 °C. (a) True thermodynamic stability constants in the presence of bicarbonate. (b) Estimates of binding affinities from both ITC and DSC data. (c) Data from Refs. [62,63] and Table 1. ΔS°, ΔG° are calculated using $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$ and $\Delta G^\circ = -RTLnK$.

In the absence of bicarbonate		n	ΔH (kJ/mol)	K_{ITC} (M^{-1})	Log K [a]	ΔG^0 (kJ/mol)	ΔS^0 (J/mol.K)	Ref.
hTf + Fe(III) [c]	C-lobe	1.0	−36.37	3.3×10^7	22.2	−43.20	22.75	[63]
	N-lobe	1.0	−23.41	3.9×10^6	21.3	−37.87	48.18	
oTf + Fe(III) [c]	C-lobe	1.0	−15.05	2.3×10^6		−36.55	71.63	[62]
	N-lobe	1.0	−31.77	2.4×10^7		−42.51	35.78	
MTf + Fe(III)	C-lobe	–	–	–	–	–	–	
	N-lobe	1.0	−35.95	2.6×10^7	17.65	−42.32	21.36	[76]
hTf + Ti(IV)	C-lobe	1.0	−8.03	3.2×10^6	26.8	−37.13	97.60	[90]
	N-lobe	1.0	−4.89	2.4×10^5	25.7	−30.71	86.60	
hTf + VO^{2+} (from $VOSO_4$)	C and N−lobes	2.0	−24.66	1.7×10^5 [b]		−29.85	17.41	[92]
				7.0×10^4 [b]		−27.65	10.03	
hTf + VO^{2+} (from BMOV)	C and N-lobes	2.0	−25.08	3.0×10^5 [b]		−31.26	20.73	[92]
				8.0×10^5 [b]		−33.69	28.88	
hTf + VO_3^- (from $NaVO_3$)	C and N-lobes	2.0	−38.04	3.1×10^5 [b]		−31.34	22.47	[92]
				3.0×10^5 [b]		−31.26	22.74	

a much stronger affinity of Tf to Ti(IV) than to Fe(III) (i.e. log $K_C = 22.7$ and log $K_N = 22.1$ for Fe(III) [91]).

5.2. Vanadium binding to hTf

A series of ITC and DSC experiments using different vanadium sources, such as vanadyl sulfate ($VOSO_4$), Bis(maltolato)oxovanadium(IV) (BMOV) and sodium metavanadate ($NaVO_3$), were used to determine the thermodynamic parameters of vanadium binding to the C-lobe and N-lobe of apo-hTf [92]. The results are summarized in Table 2. Briefly, it was found that the calorimetric traces for the three vanadium ions obtained from BMOV, $VOSO_4$ or $NaVO_3$ are similar to each other and that apo-Tf binds indistinguishably two equivalents of vanadyl/vanadate ions at the N- and C-sites. Vanadyl ions are suggested to readily oxidize to vanadate ions where the average association constant of vanadate binding to hTf is $K_{ITC} \sim 10^5\ M^{-1}$ (Table 2), a result consistent with earlier measurements of vanadate ions binding to transferrin [93].

6. Isothermal titration calorimetry studies of transferrin binding to the transferrin receptor

Extensive studies have focused on the thermodynamics and kinetics of Fe^{3+} binding to a number of transferrins. However, little attention has been given to the thermodynamic characterization of the interaction of transferrin with its receptor. A 7.5 Å cryo-EM model of the hTf/TfR complex has provided the first approximation of the regions contributing to the high binding affinity of hTf to the TfR [81]. Extensive mutagenesis work on the human TfR by the Bjorkman lab [94] in combination with radiolytic footprinting studies [95,96] validated some parts of the cryo-EM model and highlighted other areas of possible interest. The data suggested that the main contact with the helical region of the TfR is through the C1 sub-domain. Other studies [97,98] have shown that the isolated N-lobe does not bind to the TfR and/or donate iron to cells and thus are in contrast with the hTf/TfR cryo-EM model. On the other hand, the binding of the human C-lobe alone to TfR has a dissociation constant K_D of ~650 nM in cell binding experiments [99] and a K_D of ~800 nM in SPR experiments [81]. This binding is ~650–800 fold weaker in comparison to the diferric hTf which has a K_D of ~1 nM. The implication of this data is that the presence of the two lobes is critical for high receptor affinity. In order to validate or refute some of these results, ITC measurements with a number of transferrin samples were performed.

6.1. ITC of full-length Diferric (Fe_2 hTf) and Monoferric (Fe_N hTf and Fe_C hTf) transferrin interaction with TfR

At pH 7.5, apotransferrin does not have any measurable affinity for the transferrin receptor as determined by ITC (Bou-Abdallah et al., unpublished data). The heat generated from this binding interaction is similar to that of the background heat due to dilution. This result might be expected since the apoprotein does not contain any iron to deliver. On the other hand, iron-containing transferrin (whether monoferric or diferric) is predicted to interact strongly with the receptor. Fig. 1 shows the raw calorimetric data and the total heat generated upon the interaction of diferric and monoferric transferrins with the transferrin receptor at pH 7.5 [100]. Since the transferrin receptor exists as a dimer in solution, the integrated heats (Fig. 1 insets) were plotted as a function of the molar ratio of hTf to TfR monomer, thus the stoichiometry of one Tf per one TfR monomer. The strong binding affinities ($K \sim 10^7$–$10^8\ M^{-1}$) between the three transferrin samples and the transferrin receptor were achieved by both favorable enthalpy and entropy changes ($\Delta H^0 \sim -17$ to -33 kJ/mol and $\Delta S^0 \sim 50$–85 J/mol.K). The large and positive entropy values are most likely due to the change in the hydration of the two proteins upon

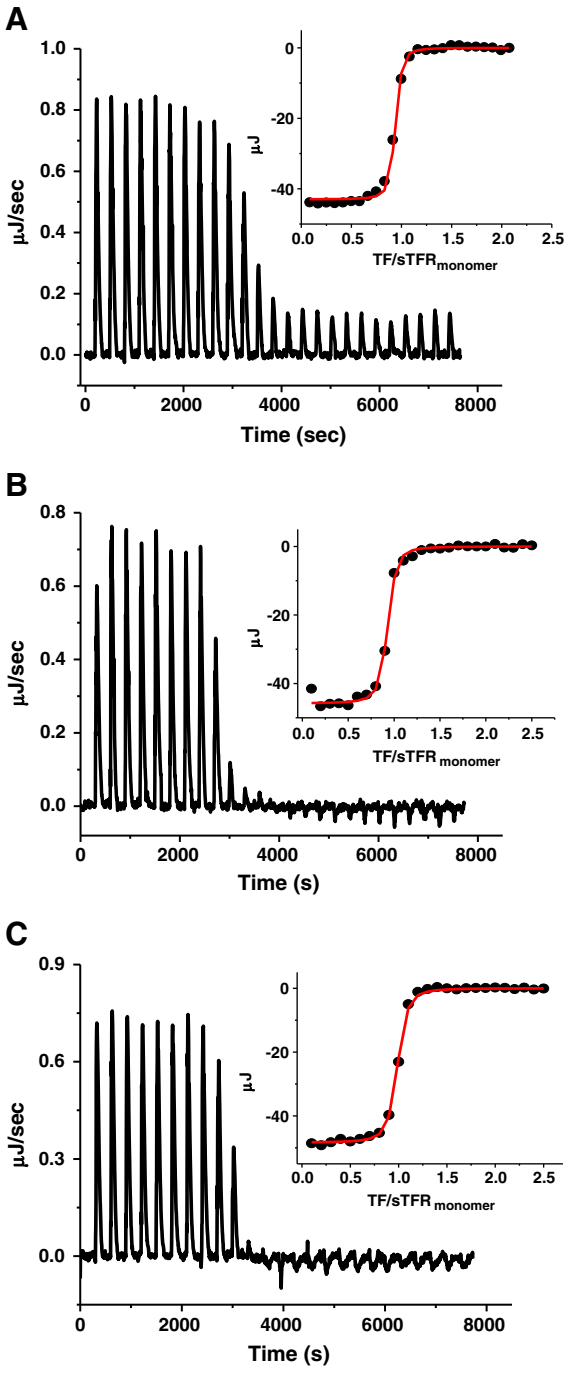

Fig. 1. ITC data for Fe_2 hTf (A), Fe_C hTf (B) and Fe_N hTf (C) titrations with TfR. Insets: plot of the integrated heat versus the Tf/TfR monomer ratio. Conditions: (A) 130 µM hTf titrated with 10 µl injections of 12 µM TfR; (B) and (C) 260 µM hTf titrated with 10 µl injections of 20 µM TfR. All protein solutions (1.8 ml hTf and 250 µl TfR) were in 100 mM Mops, pH 7.4 containing 50 mM NaCl, 50 mM $NaHCO_3$ buffer, and 25 °C. (Reprinted from Ref. [100]) with kind permission of Wiley InterScience, Copyright 2009 John Wiley & Sons, Ltd).

binding. It is interesting to note that the affinity of the diferric protein for the transferrin receptor is about 10-fold higher than either of the monoferric transferrins, and the enthalpy of binding is twice as high reflecting contributions from each lobe of transferrin. Similar affinity values were reported in an earlier study using rabbit reticulocytes [101]. The stronger binding affinity and favorable change in enthalpy are, however, compensated by an entropic penalty [100]. Earlier kinetic and thermodynamic studies using fluorescence emission T-jump spectroscopy and fluorescence

correlation spectroscopy reported comparable affinity values [33,102].

6.2. ITC of Diferric (Fe_2 hTf) N-lobe mutants interaction with TfR

Several amino acid residues in the N-lobe of human transferrin (N1 and N2 sub-domains) were identified in the "cryo-EM model" [80] and "epitope mapping studies" [103] and were proposed to interact with the helical region of the TfR. In order to elucidate which of these residues was crucial to Tf–TfR recognition, several single point N-lobe mutants (P142A, R143A, K144A and P145A) in a diferric Tf background were titrated against the transferrin receptor. The ITC results are presented in Fig. 2 [100]. The ITC data corroborated the prediction from the cryo-EM model and showed that *only* three of the four residues (P142, K144 and P145) appear to be involved in the interaction with the TfR. These results confirm earlier work [97–100,103,104] implicating the N-lobe in this interaction. Thus, at neutral pH, it appears that both lobes are required for the strong binding affinity of the diferric Tf to TfR [100].

More recent ITC measurements of different iron-free and iron-containing transferrin samples using a Nano ITC low volume calorimeter from TA Instruments showed somewhat lower binding affinities but similar heats of reactions (Bou-Abdallah et al., unpublished data). While the data in reference [100] was collected on an older ITC instrument (a CSC Model 4200 isothermal titration calorimeter from Calorimetry Science Corporation), the exact reason(s) for the lower binding affinities are currently unknown. Nonetheless, the data from either instrument exhibit a similar overall trend whereby monoferric C-lobe or N-lobe transferrins consistently show lower binding affinity to the receptor in comparison to diferric transferrin. It is noteworthy that the Nano ITC low volume instrument uses much less sample than the CSC model (5 to 6 times less protein, i.e. 0.4–0.5 mg Tf and TfR is required per ITC experiment on the Nano ITC vs 2–3 mg for the CSC model). Thus, while the CSC experiments are impacted by large amounts of Tf and TfR, the Nano ITC has the dual benefit of using less protein while avoiding solubility issues at higher protein concentrations.

6.3. ITC of transferrin binding to the transferrin receptor from Neisseria meningitidis

A recent investigation of the binding of human transferrin to the transferrin receptor of *Neisseria meningitidis* was carried out using microcalorimetry [105]. The *N. meningitidis* receptor consists of two types of subunits, a transmembrane protein, TbpA, proposed to serve as a channel for the transport of iron across the outer-membrane-anchored protein, TbpB, which interacts with TbpA and is itself composed of two different isotypes, TbpB I and TbpB II [105]. The two types of receptor proteins are known to bind hTf but conflicting results regarding the affinity, modes of binding, role and contribution of each protein subunit to hTf binding, and consequently iron uptake, have emerged [105 and references therein]. The ITC data cleared much of this controversy and allowed a detailed characterization and clear assessment of the thermodynamics of this interaction. In brief, the data revealed three different and independent binding sites for holo-hTf on the full receptor (TbpA/TbpB) whereby TbpA contained the high

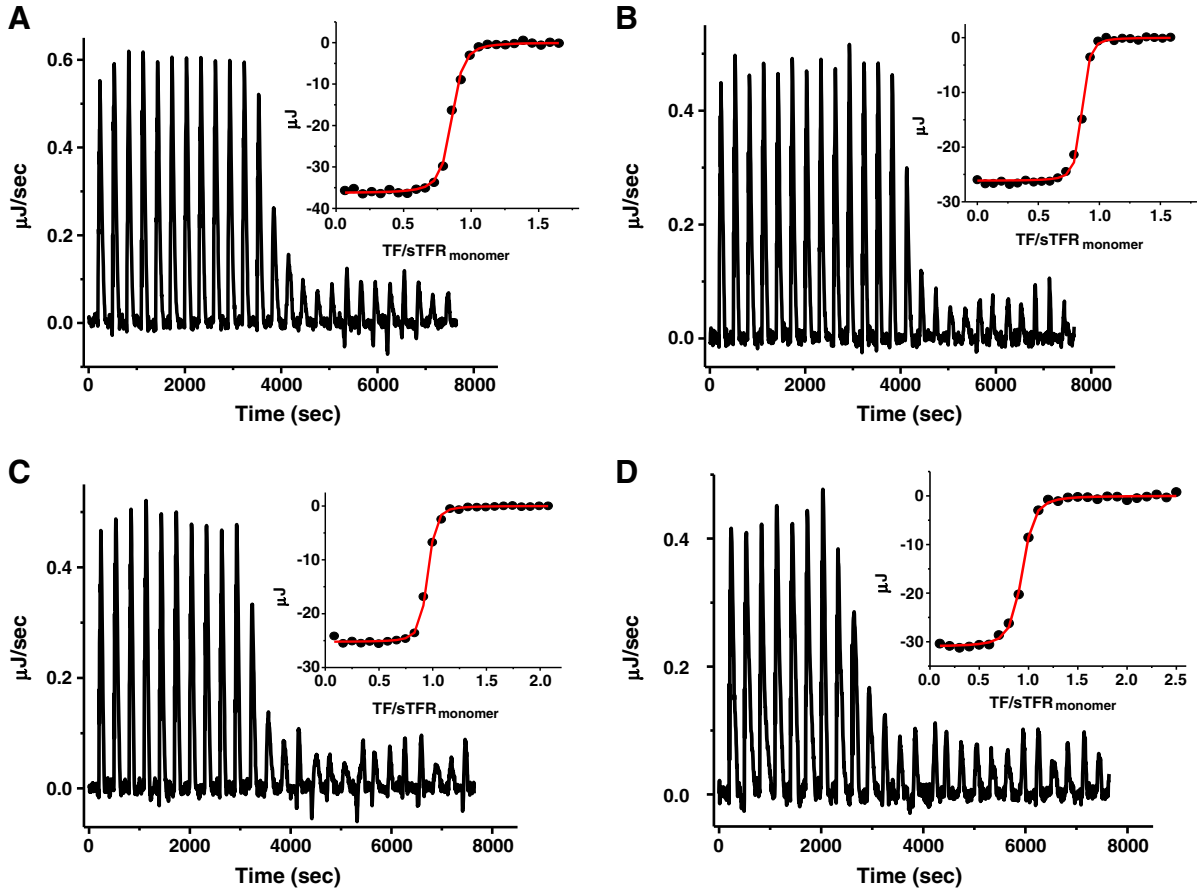

Fig. 2. ITC data for (A) Fe_2 hTf-P142A, (B) Fe_2 hTf-R143A, (C) Fe_2 hTf-K144A, and (D) Fe_2 hTf-P145A titrations with TfR. The experimental conditions are the same as in Fig. 1. (Reprinted from Ref. [100] with kind permission of Wiley InterScience, Copyright 2009 John Wiley & Sons, Ltd).

affinity site (n ~ 1 and K_D ~ 0.7 nM) while TbpB comprised the other two lower affinity sites (n ~ 2 and K_2 ~ 22.2 nM). The binding of hTf to the isolated subunits (TbpA and TbpB) was also demonstrated whereby TbpA exhibited a strong preference for apo-hTf (20 times higher affinity than holo-hTf) whereas TbpB had a preferential affinity to holo-hTf. Overall, the ITC data provided new insights into the structure and function of the transferrin receptor from *Neisseria meningitidis* and showed that TbpB controls the binding specificity of TbpA towards hTf by increasing the local concentration of holo-hTf thus affecting the iron uptake capacity of the receptor [105].

7. Conclusions and future studies

The use of ITC in characterizing biomolecular interactions has been steadily increasing since the development of a new generation of sensitive calorimetry instruments [59,61]. Despite the existence of some limitations (i.e. restricted range of binding affinities, low heat of binding reaction and solubility), the ability to perform a simple titration and collect a full set of thermodynamic parameters is a major advantage of this direct and true in-solution method. Thermodynamic data provide extremely useful information on the energetic forces that drive a biomolecular interaction but the challenge remains in being able to properly analyze thermodynamic results and identify links between structure and function. Here, we have reviewed important ITC data on the interaction of metal ions with transferrin and outlined the thermodynamics of the transferrin–transferrin receptor system at physiological pH. As mentioned earlier, the iron loaded transferrin delivers its content of iron by receptor-mediated endocytosis after attaching to the transferrin receptor at the cell membrane. At the acidic pH of the endosome, the apo-protein remains attached to its receptor before it is returned back to the cell surface for another cycle. Thus, future ITC experiments must be performed to elucidate the thermodynamics of this interaction at the acidic pH of the endosome and subsequently evaluate the contributions of specific amino acid residues on both proteins and their role in iron delivery to cells.

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