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Contrasting Synergistic Anion Effects in Vanadium(V) Binding to Nicatransferrin versus Human Serum Transferrin[†]

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ABSTRACT: Some ascidians sequester vanadium and other metal ions that are bound and transported in higher organisms by transferrin. The ascidian *Ciona intestinalis* has a monolobal transferrin (nicatransferrin) in its plasma. The binding of vanadium(V) to nicatransferrin was investigated by using isothermal titration calorimetry and UV−vis spectroscopy, in the presence and absence of $NaHCO_3$, and was compared with human serum transferrin. Nicatransferrin and serum transferrin bind V(V) with similar strengths [$K = (2.0 \pm 0.6) \times 10^5$ M^{-1} for nicatransferrin]; however, nicatransferrin requires a synergistic anion for V(V) binding, whereas serum transferrin does not. Spectroscopy supports a different kind of coordination site.

The transferrins (Tfs) make up a group of proteins whose primary function is to sequester and transport iron (*1*). Serum transferrin (sTf) is the most widely studied of this class and is found primarily in the circulatory and lymphatic systems of higher organisms such as vertebrates. Serum transferrin is an 80 kDa bilobal protein comprised of two homologous lobes (N and C lobe). Each lobe binds one Fe(III) with two tyrosines, an aspartate, a histidine, and an exogenous synergistic anion, usually carbonate (*1*, *2*).

Transferrin binds a variety of metal ions besides Fe(III), which makes it an attractive delivery vehicle for metal-based medicines (*3*), including ones featuring vanadium (*4*). Vanadium(V) does not require a synergistic anion for binding to hsTf (*5*). Instead, $[V(V)O_2]^+$ binds in place of $[Fe(III)(CO_3)]^+$ with the V(V) most likely coordinated to two deprotonated Tyr residues (Figure 1) (*6*). The involvement of an arginine side chain important for positioning the carbonate during Fe(III) binding has been invoked.

The internal homology of sTfs suggests that these bilobal proteins evolved from a gene duplication and fusion of a primitive monolobal form (*7*, *8*). Monolobal transferrins have been identified only in ascidians, marine invertebrate chordates on the evolutionary boundary between vertebrates and invertebrates (*9*). They are well studied by evolutionary biologists and have intrigued inorganic chemists for nearly 100 years, since they were reported to sequester high concentrations of metals such as vanadium from their environment (*10*, *11*).

The molecular mechanism of vanadium transport in ascidians is not fully understood. Early work suggested that vanadium enters the ascidian in the +5 oxidation state and that V(V) in the blood plasma might exist as the free ion or be weakly bound by an unspecified plasma protein (*12*). It might be reduced once bound to protein. More recent studies suggest V(V) is reduced in the blood cells by a vanadate reductase (*13*), perhaps vanabin2 which reduces V(V) to V(IV) in the presence of glutathione (*14*).

The genome of the ascidian *Ciona intestinalis*, a moderate vanadium accumulator (*11*, *12*), was sequenced (*15*), revealing genes for both a monolobal and a bilobal Tf. The 37.7 kDa monolobal Tf (nicatransferrin or nicaTf) was isolated from the plasma of *C. intestinalis* (*16*). Further work with recombinant nicaTf revealed that it binds one Fe(III) with a binding constant ($K = 3 \times 10^{18}$ M^{-1}) which is much weaker than that for binding of Fe(III) to human serum transferrin (hsTf) ($K = 1-6 \times 10^{22}$ M^{-1}) (*17*).

Given the status of *C. intestinalis* as a vanadium-sequestering species and the possible role of Tfs including nicaTf in vanadium transport, we investigated the thermodynamic and spectroscopic properties of binding of V(V) to nicaTf. Isothermal titration calorimetry (ITC) (*18*) and UV−vis spectroscopy were used to characterize binding.

In the ITC experiments, ammonium metavanadate (NH_4VO_3) was titrated into recombinant nicaTf at 27 °C (see the Supporting Information for experimental details). The NH_4VO_3 concentration (1 mM) in the titration syringe was close to the concentration above which polynuclear species occur at pH 7.4 (*19*). However, control titrations in the absence of protein gave no suggestion of heats accompanying dissociation into monomers (Figure SI1), and importantly, experiments for hsTF employing 100 μM NH_4VO_3 in the titration syringe gave experimentally indistinguishable results. Also, similar concentrations of vanadate were used in previous ITC (*4*) and other (*5*) studies of binding of vanadium to transferrins. HEPES was chosen as the buffer because, of the common biological buffers, HEPES has the weakest interaction with vanadates (*20*). In concord with this point, experiments employing Tris as the buffer yielded irreproducible results (data not shown). Sodium bicarbonate ($NaHCO_3$), when present, was in both the titration syringe and the cell. Conditions such as temperature and initial concentrations were chosen following earlier calorimetric work on binding of Fe(III) to ovotransferrin and sTf (*21*, *22*). Each experiment was performed at least in quadruplicate using different protein preparations; error bars reflect standard deviations across no fewer than four experiments. Errors in the fits to each individual data set were much smaller than standard deviations across replicates (see the Supporting Information).

Binding of V(V) to hsTf is a well-studied system that we used as a benchmark for binding of V(V) to nicaTf (*4*, *5*, *23*). Bicarbonate was included in previous ITC work (*4*), though it is not required for V(V) binding (*5*). Here experiments with and without bicarbonate were performed for hsTf (Figure SI1). The data

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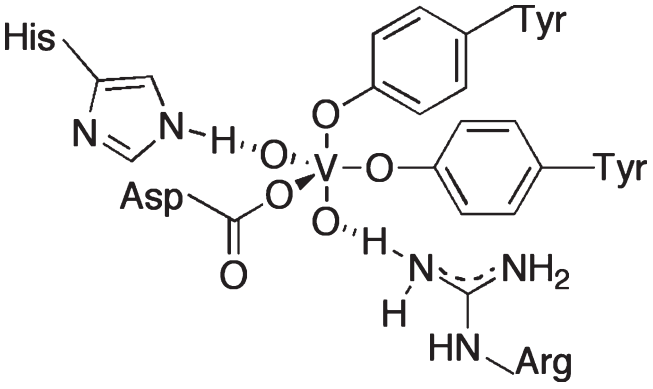

FIGURE 1: Model for V(V) binding as VO_2^+ to the N lobe of human serum transferrin adapted from ref *6*, consistent with data from earlier studies (*5*).

confirmed by ITC the previous reports that bicarbonate is not required for binding of V(V). In control experiments in the absence of protein, the endothermic heats of dilution were reproducibly slightly larger in the absence of bicarbonate (Figure SI1). This finding may reflect the existence of carbonate complexes of vanadate, which have been postulated (*24*). The fit parameters for vanadate−hsTf binding in the presence of $NaHCO_3$ were in good agreement with previous ITC work, which was published while our experiments were in progress (*4*). The measured binding constants were generally higher in the absence of bicarbonate than in its presence (Figure SI1 and Table SI1). Vanadate binding was entropically favored in the presence of bicarbonate but disfavored in its absence. This effect was overshadowed by enthalpically more favorable binding in the absence of bicarbonate. Taken together, these results suggest competition of carbonate for the metal binding site of hsTf and/or for the vanadate itself, or two V(V) complexes for hsTf, one with carbonate and one without.

Experiments with nicaTf yielded unexpected results. No V(V) binding to nicaTf was observed in the absence of bicarbonate (Figure 2). The fact that nicaTf requires a synergistic anion for V(V) binding sharply contrasts with the case of hsTf. The requirement for a synergistic anion for vanadium binding may be related to the replacement of the analogue of Arg124, a critically important residue for synergistic anion binding in sTf, with a Lys in nicaTf. For hsTf, the R124K mutant retains its ability to bind iron, although the release is moderately faster (*25*). The Arg versus Lys difference in nicaTf is the only proposed metal binding site residue that differs between hsTf and other bilobal Tfs. This difference in ascidians has been suggested to confer a functional advantage in cold dwelling species (*26*). The change, although conservative, may help explain the difference in strength and preference for synergistic anion upon V(V) binding. When Fe(III) is bound to nicaTf, there is no evidence of binding of V(V) (Figure SI2), suggesting that they share a binding site.

The experimental stoichiometries were revealed by ITC. The binding stoichiometry for the V(V)−hsTf titration in the presence of bicarbonate was 1.8 ± 0.2 (Table 1) which is within experimental error of the expected value of 2 to account for one vanadium ion binding to each lobe. However, previous work using ^{51}V NMR also determined values close to 1.8 (*27*, *28*). For nicaTf, the observed stoichiometry (1.2 ± 0.3) agrees well with the expected value for binding at one site in the monolobal protein. It is also in agreement with earlier

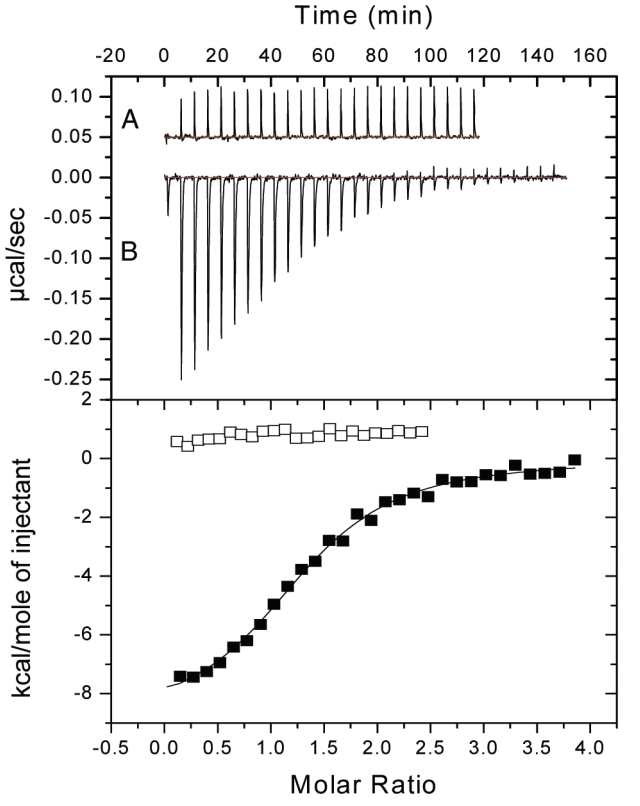

FIGURE 2: Raw ITC data analysis (top) for titration of (A) 1.0 mM NH_4VO_3 into 50 μM nicatransferrin in the absence of $NaHCO_3$ and (B) 1.0 mM NH_4VO_3 into 40 μM nicatransferrin in the presence of 25 mM $NaHCO_3$. The bottom panel shows integrated heat data, fit to a one-site binding model. White squares correspond to data from trace A, and black squares correspond to data from trace B. All solutions were in 100 mM HEPES (pH 7.4).

Table 1: Thermodynamic Parameters for ITC Characterization of Binding of V(V) to nicaTf and hsTf in 100 mM HEPES and 25 mM sodium bicarbonate (pH 7.4) at 27 °C

protein	n	K_{ITC} (M^{-1})	ΔH (kcal/mol)	ΔS (cal mol^{-1} K^{-1})
nicaTf	1.2 ± 0.3	(2.0 ± 0.6) × 10^5	−8.8 ± 0.7	−5.2 ± 2.6
hsTf	1.8 ± 0.2	(1.6 ± 1.3) × 10^6	−7.3 ± 2.3	3.8 ± 6.4

work on the stoichiometry of binding of V(V) to nicaTf using dialysis (*17*).

Vanadium(V) binding is exothermic and enthalpically driven for nicaTf (ΔG = −7.3 kcal/mol, and ΔH = −8.8 kcal/mol). Binding is slightly tighter than anticipated for an ascidian plasma V(V) binding protein, projected to have a binding constant of $\leq 10^4$ (*12*). The contribution from $-T\Delta S$ is slightly unfavorable (1.5 kcal/mol). Vanadate binding to hsTf in the presence of bicarbonate also has most of the driving force (ΔG = −8.5 kcal/mol) coming from ΔH (−7.3 kcal/mol) but is slightly less enthalpically favorable. The latter reaction has a small favorable entropic contribution ($-T\Delta S$ = −1.1 kcal/mol). This contribution may reflect displacement of CO_3^{2-} from the hsTf binding site and/or from vanadate (see above). Protein conformational changes upon metal binding may contribute. Across three to five replicates using different preparations for both nicaTf and hsTf, the binding constant for binding of V(V) to nicaTf in the presence of bicarbonate differed by at most 1 order of magnitude. This result contrasts with the case of Fe(III), for which binding

to nicaTf is 4 orders of magnitude weaker than to hsTf (*17*). These conditional binding constants determined using ITC can only be compared to values measured under the same solution conditions.

UV−vis spectroscopy further characterized the binding of V(V) to nicaTf in the presence of bicarbonate. The lack of spectral changes upon V(V) binding (Figure SI3) reflects a difference in the ligands or geometry of the nicaTf metal binding site compared with that of hsTf (Figure SI4). Binding of V(V) to hsTf results in a ligand to metal charge transfer (LMCT) at 260 nm (ε = 9400 M^{-1} cm^{-1}) that supports V(V) coordination as VO_2^+ to deprotonated Tyr residues in the metal binding site (Figure 1) (*5*).

The lack of an intense LMCT for the V(V)−nicaTf complex strongly suggests that V(V) does not bind as V^{5+}, VO^{3+}, or VO_2^+ to deprotonated Tyr in nicaTf. This result could be because the bound moiety is anionic, because oxo or hydroxo ligands preclude Tyr binding, and/or because carbonate (or other) ligands intercede. Rather than the cationic forms above, vanadium(V) may bind as $H_2VO_4^-$ or HVO_4^{2-}, species that dominate the aqueous speciation of V(V) at this pH (*19*). Alternatively, considering the requirement for $NaHCO_3$, V(V) may bind as a complex of carbonate such as $[VO_2(CO_3)_2]^{3-}$ or $VO_2(CO_3)^-$ (*24*). Each of these is an anionic moiety (unlike the cationic VO_2^+ or $[FeCO_3]^+$ that binds to hsTf) and would not lower the pK_a of Tyr, hindering its deprotonation and coordination to V(V).

This difference in spectroscopy between nicaTf and hsTf is reminiscent of their properties upon Fe(III) binding. For nicaTf, there is no charge transfer near 465 nm as would be expected for a Tyr to Fe(III) charge transfer (*17*). The related case of binding of Ti(IV) to hsTf may be relevant, in which hydrolytic binding of water-derived ligands (oxo or hydroxo) has been invoked to explain the loss of the Tyr to Ti(IV) LMCT at elevated pH, even though metal binding is preserved (*29*).

Binding of anionic moieties may connect this primitive monolobal transferrin to the distantly related anion binding proteins, or to the bacterial periplasmic binding protein NikA, which binds metal not as the naked cation but as a metallophore chelate (*30*). The metal binding site of nicaTf is more accessible than that of hsTf: both native (*16*) and recombinant (data not shown) nicaTf are retained on an Fe(III)·NTA column, whereas hsTf is not. This result supports the idea that nicaTf can bind metal ions in chelated form.

NicaTf binds V(V) about as strongly as hsTf does, supporting the hypotheses that the ascidian monolobal transferrins may have evolved as more general metal ion transporters and may contribute to ascidian vanadium trafficking. The unexpected requirement for sodium bicarbonate and the distinct spectroscopy upon V(V) binding further distinguish nicaTf from other characterized transferrins. The carbonate requirement for binding may further afford a control or triggering mechanism (*31*).

SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures, four figures, and one table. This material is available free of charge via the Internet at http://pubs.acs.org.

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