Contrasting Synergistic Anion Effects in Vanadium (V) Binding to Nicatransferrin Versus Human Serum Transferrin

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Reagents and Chemicals

Apo-human serum transferrin (low endotoxin grade, from human plasma) was obtained from Calbiochem and Sigma and used without further purification. Ammonium metavanadate (NH_4VO_3) was purchased from Sigma Aldrich. Water (18.2 mΩcm) was obtained from a Barnsted Nanopure purification system (model D11931) and was used to prepare all solutions.

Solution Preparation

All glassware was demetalated by soaking in a Citranox (Alconox) bath and rinsed thoroughly with water. Buffers and added buffer components included: sodium acetate (J.T Baker), ethylenediamine tetraacetic acid disodium salt (99% J.T Baker), 4-hydroxyethyl-1-piperazine ethanesulfonic acid (HEPES) (American Bioanalytical), sodium bicarbonate (Mallinckrodt Chemicals) and sodium chloride (J.T Baker). Solutions that were bicarbonate free were bubbled gently with N_2 prior to use. All protein was dialyzed in 10 kDa mwco dialysis tubing (Spectra/Por). If needed, protein was concentrated by using a 10 kDa Amicon centrifugal concentrator (Centriprep). All pH measurements were made on a Thermo Orion model 410 A plus with a Orion Ross electrode. The pH was calibrated by using standard pH 4.0, 7.0 and 10.0 buffers.

Protein Expression and Purification

Nicatransferrin (nicaTf) was expressed in the yeast *Pichia pastoris* (Invitrogen) and purified following the procedure from Tinoco et al. (*1*). Nicatransferrin was further purified by using size exclusion chromatography on a Superdex-75 10/300 GL column and was eluted in 50 mM HEPES, 0.15 M NaCl (pH 7.4) on an ÄKTA prime column

chomotography apparatus. The fractions that contained pure nicatTf were identified by SDS/PAGE stained with Coomassie brilliant blue. Protein concentration was determined from A_{280} measurements using an experimental extinction coefficient of 75,000 $M^{-1}cm^{-1}$ measured at the Molecular Structure Facility at UC Davis and agreed well with protein concentrations determined using the Bradford assay. UV spectra were recorded on a Cary Bio50 UV/vis spectrophotometer. Apo-nicaTf was prepared by dialyzing the protein against 0.1 M sodium acetate, 10 mM EDTA, pH 5.5 buffer (*1*). The proteins were then dialyzed in 100 mM HEPES (pH 7.4) in the presence or absence of 25 mM sodium bicarbonate. For experiments in the absence of bicarbonate, the dialysis buffer was bubbled gently with N_2 before and during dialysis to ensure the removal of dissolved CO_2. The protein was then used for ITC experiments. Iron loaded nicaTf was prepared by dialyzing the protein in 100 mM HEPES, 25 mM sodium bicarbonate, 1 mM $Fe(NTA)_2$ (NTA = nitrilotriacetic acid) (1). Excess iron was removed with further dialysis in 100 mM HEPES, 25 mM sodium bicarbonate, pH 7.4 buffer.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was performed on a MicroCal ITC_{200} (Microcal Inc.). Experiments were performed at 27° C by titration of NH_4VO_3 (typically 1 mM) into protein (typically 50 – 80 μM) both in the same buffer, 100 mM HEPES pH 7.4, in the presence or absence of 25 mM sodium bicarbonate. HEPES buffer was used for experiments because it does not interact strongly with V(V) (2). Control experiments were performed by titration of NH_4VO_3 into protein-free buffer. Titration of NH_4VO_3 into Fe (III)-nicaTf was performed at 27° C in 100 mM HEPES, 25 mM sodium bicarbonate, pH 7.4. The result of this experiment, which shows no binding of V(V) to the Fe(III)

bound nicaTf (Figure SI3) suggests that Fe(III) and V(V) in nicaTf most likely share the same binding site. The ITC data were corrected for the heat of dilution of the titrant by subtracting the control data from the experimental data by using Origin 7.0. Binding stoichiometry, binding constant, entropy and enthalpy were determined by fitting the data to one type of site. Fitting to a model with one type of binding site (with variable stoichiometry and thermodynamic parameters) was adequate to fit the data; the data did not support the inclusion of additional sites.

The errors in Table 1 of the main text are reported as standard deviations across four replicate trials in the case of human serum transferrin with different protein preparations being used for each experiment. There was sample variability across different suppliers of human serum transferrin, which included Sigma and Calbiochem. For nicatransferrin, the data presented are averages and standard deviations among five experiments on different protein preparations. The values presented in Table SI1 are the fit parameters for one data set (in Figure SI1) fit to a one site binding model using Origin 7.0 as described above. The errors shown represent the errors for the fit of those data only. Errors in fits to each individual data set were much smaller than standard deviations across replicates.

UV/vis Experiments

UV/vis experiments were performed on a Cary Bio50 UV/vis spectrophotometer. A solution of 500 μM NH_4VO_3 was added in 1 μL aliquots to 600 μL of 8 μM nicaTf. Both the vanadium and protein solution were in 100 mM HEPES, 25 mM sodium bicarbonate, pH 7.4. The spectra were recorded from 200-800.

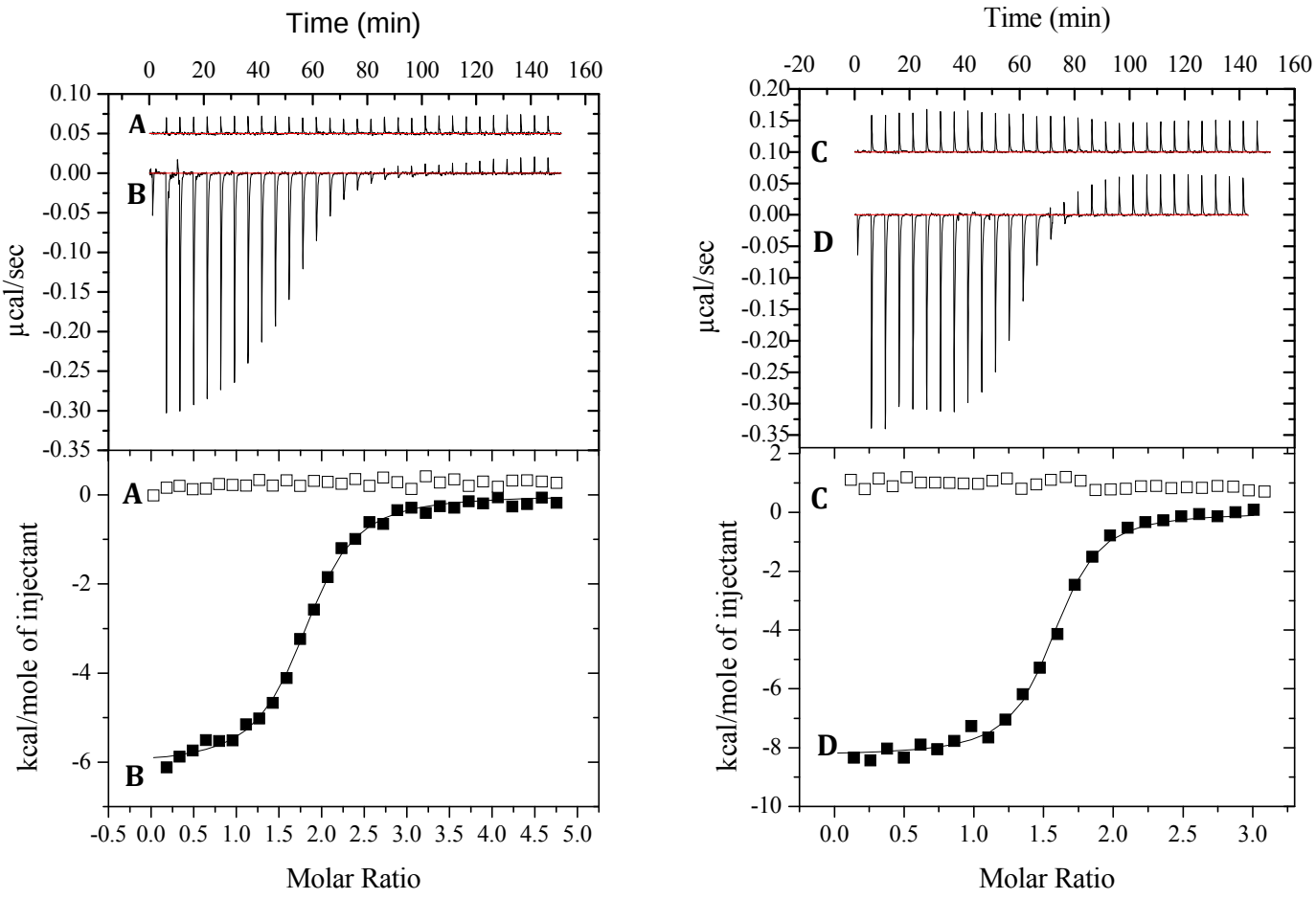

Figure SI1. Binding of V(V) to hsTf in the presence and absence of bicarbonate. A) 1.0 mM NH_4VO_3 into buffer in the presence of bicarbonate B) 1.0 mM NH_4VO_3 into 43 µM hsTf in the presence of bicarbonate C) 1.0 mM NH_4VO_3 into buffer in the absence of bicarbonate D) 1.0 mM NH_4VO_3 into 36 µM hsTf in the absence of bicarbonate

Table SI1. Fit parameters for data shown above in Figure SI1. The data presented below are the fits of the data in Figure SI1 to one type of site using Origin 7.0.

Sample	n	K_{ITC} (M^{-1})	ΔH (kcal/mol)	ΔS (cal/mol ·K)
HsTf + bicarbonate	1.78 ± 0.01	5.3 ± (0.4) x 10^5	-6.1 ± 0.6	6.0
HsTf - bicarbonate	1.53 ± 0.10	6.0 ± (0.4) x 10^6	-13.1 ± 0.5	-12.5

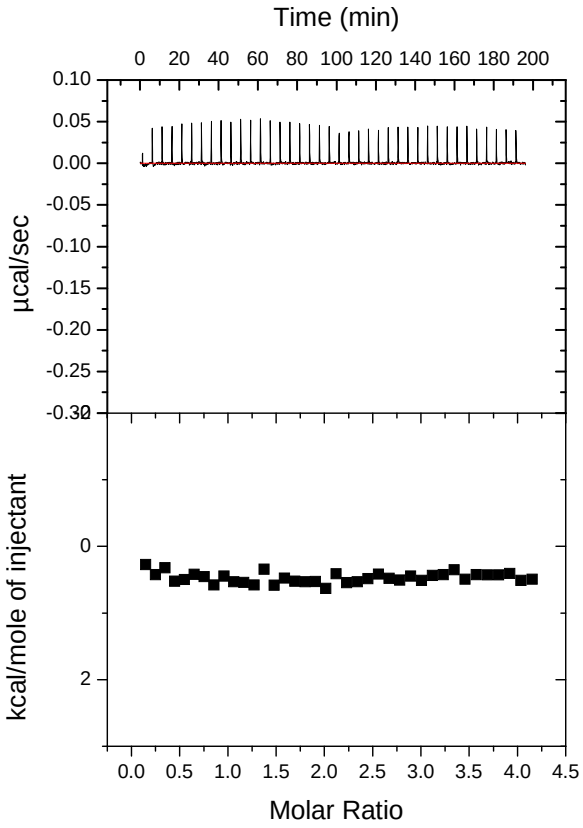

Figure SI2. Titration of 1.0 mM NH_4VO_3 into 52 µM Fe (III)-nicaTf in 100 mM HEPES, 25 mM sodium bicarbonate, pH 7.4, 27 ºC.

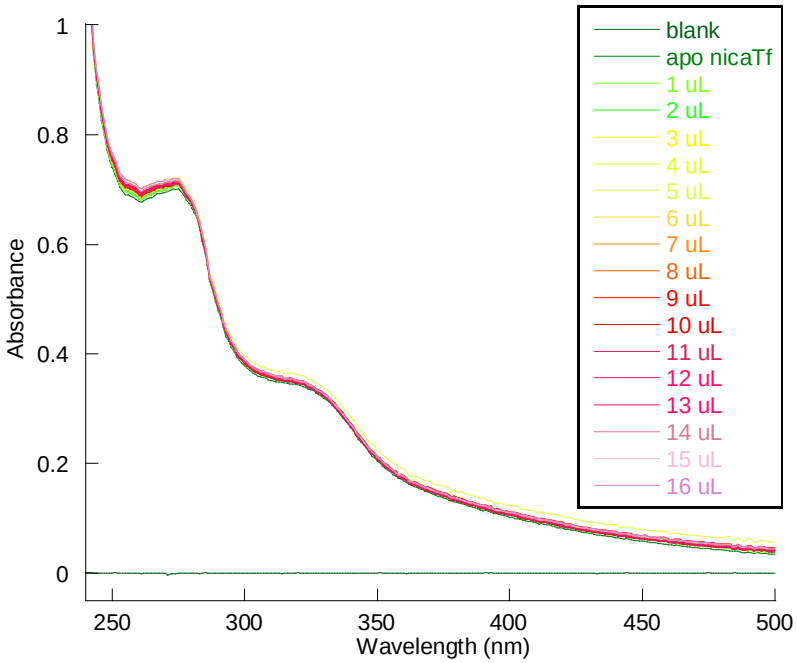

Figure SI3. UV/vis titration of 500 µM NH_4VO_3 into 600 µL of 8.7 µM nicaTf.

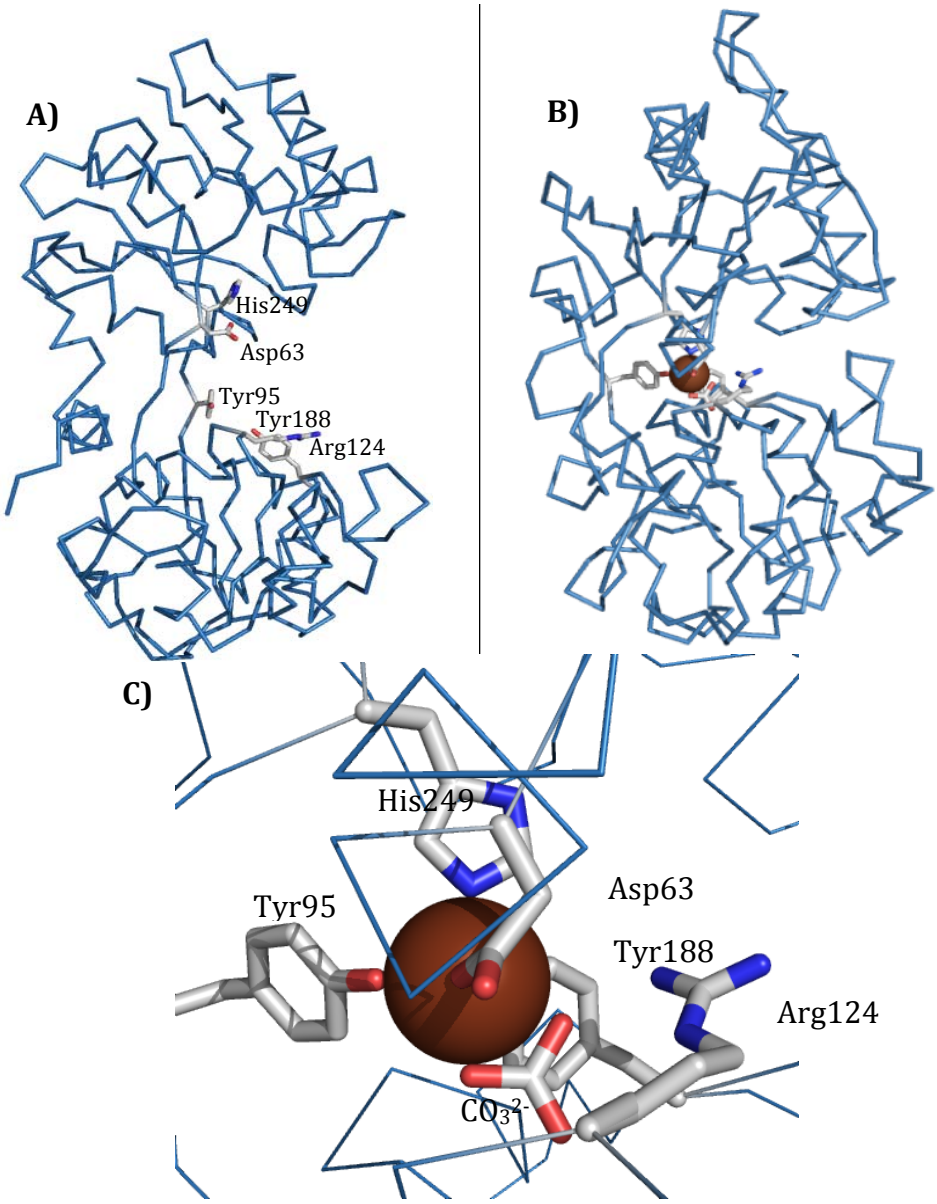

Figure SI4. Structures of the apo- and Fe-bound N-lobe of human serum transferrin, highlighting the iron ligands (Asp63, Tyr95, Tyr188, and His249) and an important carbonate-positioning residue (Arg124) (2,3). The N1 domain (residues 1-93 and 247-315) is on top and the N2 domain (residues 94-246) is on bottom. Iron ligands Asp63 and His249 are contributed by domain N1, iron ligand Tyr95 is contributed by the hinge region between domains, and iron ligand Tyr188 as well as Arg124 are contributed by domain N2. (A) Wild type apo-transferrin from coordinates PDB 2HAV, truncated to show only the N-lobe (residues Lys4-Cys331) (2). (B) Conformation A for the structure of the Fe-bound N-lobe from coordinates PDB 1A8E (3). (C) Expanded view of the active site in the same orientation as (B). The figure was prepared by using PyMol.

References:

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