DOI: 10.1002/ejic.200600314

Binding Constant of $V^{IV}O$ to Transferrin

Tamás Kiss,*[a,b] Tamás Jakusch,[b] Saâd Bouhsina,[c] Hiromu Sakurai,[d] and Éva Anna Enyedy[a]

Keywords: $V^{IV}O$–Transferrin complexes / EPR spectroscopy / UV spectroscopy / Stability constants

The interactions of bis-$V^{IV}O$-1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone (DHP) with apotransferrin (apoTF) were followed by means of electron paramagnetic resonance spectroscopy (EPR) at room temperature and at liquid nitrogen temperature. Partial ligand displacement between DHP and apoTF was observed, and the reaction was used to determine the binding constant of $V^{IV}O$ to apoTF. A value of $\log K = 14.3 \pm 0.6$ was obtained for the binding of the first $V^{IV}O$ to apoTF. In order to confirm the validity of the binding constant, UV spectroscopy was used to monitor the displacement reaction between $V^{IV}O$–apoTF and nitrilotriacetic acid (NTA). The difference between the binding constants obtained by the two different methods (ca. one log unit) was explained in terms of the possible ternary complex formation between $V^{IV}O$–apoTF and the displacing ligand. The binding constants obtained exhibit a reasonably good agreement with the linear free energy relationship (LFER) estimation.

Introduction

There is considerable interest in the binding strength of metal ions to human serum transferrin (TF) because of its natural, therapeutic, diagnostic and toxicological importance.[1] The serum transport protein TF has two very similar metal binding sites (C-lobe and N-lobe), and their affinities for metal ions differ only slightly. Each lobe contains a distorted octahedral site consisting of two Tyr-O^- anions, one His-N group, one Asp-COO^- anion and one usually bidentate hydrogen carbonate anion (the synergistic anion).[2,3] Normally, the two stepwise binding constants ($\log K$ values) differ by approximately one unit, which is beyond the purely statistical factor of four (0.6 log unit), because of a slight difference in the intrinsic binding affinities of the two lobes. The mode of binding of metal ions at the two sites depends upon the nature of the metal ion, the synergistic anion and the pH, but binding is generally a little more preferred at the C-terminal site than at the N-terminal site.[2]

[a] Department of Inorganic and Analytical Chemistry, University of Szeged,
P. O. Box 440, 6701 Szeged, Hungary
Fax: +36-62-420505
E-mail: tkiss@chem.u-szeged.hu
[b] Biocoordination Chemistry Research Group of the Hungarian Academy of Sciences,
P. O. Box 440, 6701 Szeged, Hungary
[c] Laboratoire Interdisciplinaire en Sciences de l'Environment, Université du Littoral Côte d'Opale, ELICO ESA 8013,
32 Avenue Foch, 62930 Wimereux, France
[d] Department of Analytical and Bioinorganic Chemistry, Kyoto Pharmaceutical University,
5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan

A number of experimental techniques have been used to determine the stability constants of metal–TF complexes. Difference UV spectroscopy, based on the production of new absorption bands at ca. 240 nm and ca. 295 nm[2] on the complexation of metal ions to the phenolic groups of the Tyr residues, has been applied most frequently.[4–9] Other methods include equilibrium dialysis, EPR[5] and multinuclear NMR spectroscopy,[10] the latter two also being used to distinguish the modes of metal ion binding between the two sites.[11–14]

Interest in the biochemistry of vanadium has increased enormously in the past twenty years partly because of its insulin-mimetic or more precisely insulin-enhancing properties.[15] It has been proposed that the transport of vanadium in higher organisms is mediated by TF,[16] and it was recently proved that most of the vanadium in the serum is bound to TF.[17,18] The complexation of vanadium to TF in oxidation states of +3,[19–22] +4[13,14,20–27] or +5[21–24,28–30] has been extensively studied by various research groups. The results uniformly indicate that two vanadium ions are bound to the metal-ion-free apoTF, at the usual specific Fe^{III} binding sites. Using X-band and Q-band EPR spectroscopic methods, Chasteen et al.[13,14] distinguished three $V^{IV}O$ binding environments at a physiological pH (ca. 7.4), all having similar O-donor-rich environments, but with slightly different conformations. One possibly has the normally preferred square-pyramidal or square-bipyramidal $V^{IV}O$ geometry, in which the $V^{IV}O$ oxygen atom occupies an apical position (A environment), while for the other two (B_1 and B_2) [although in-plane anisotropy (rhombic spectra) is not observed] the noncoincidence of the magnetic axes suggests significant distortion around the $V^{IV}O$ ar-

rangement. The relative intensities of the A and B resonances are strongly pH dependent. The two interconvertible A and B conformational states are believed to belong to the C-terminal and N-terminal binding sites of the protein, respectively.[31]

The binding of $V^{IV}O$ to TF is strong, and thus only stoichiometric formation of the complexes can be observed.[13,14,21] We are not aware of any attempt to characterise the complexation of $V^{IV}O$ with TF in a quantitative way; only the competition between $V^{IV}O$ and Fe^{II} binding to ferritin[26] and the equilibrium distribution of $V^{IV}O$ between albumin and TF have been studied.[24] Sun et al.[2] suggested that the LFER between the binding constants ($\log K_1$) of metal ions to the first lobe of TF and the stability constants, $\log K_{OH^-}$ for the binding of hydroxide, can be used for the prediction of unknown stability constants of metal-ion–TF complexes. Other possible correlations between the metal ion binding to TF and small N,O-donor ligands such as nitrilotriacetic acid (NTA), oxalate, acetate, glycine, malonate and lactate were also examined but none of them were as good as that with OH^-. This suggests that the two Tyr moieties at the metal-binding site of TF play predominant roles in determining the strength of the metal binding.[2] We used the above approach in our previous work in order to describe the speciation of various insulin-mimetic $V^{IV}O$ complexes in blood serum,[32] and obtained a value of $\log K_1(TF) = 13.2 \pm 1.6$ for $V^{IV}O$ binding to TF. During the study EPR spectroscopy revealed a partial displacement of apoTF by one of the low-molecular-mass carrier ligands, 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone (DHP). It was decided to make use of this reaction to assess the binding of $V^{IV}O$ to apoTF.

Accordingly, in this paper we report, to the best of our knowledge, the first binding constants of $V^{IV}O$ to human serum TF obtained by means of EPR and UV spectral monitoring of the ligand displacement reactions between the $V^{IV}O$ complexes formed with apoTF and DHP or NTA.

Results and Discussion

The EPR spectra of the $V^{IV}O$–apoTF system at a metal-ion-to-ligand ratio of 1:1 at pH 7.5 at room temperature and at 77 K (LNT: liquid nitrogen temperature) are depicted in Figure 1. In agreement with the earlier result,[33] the room temperature spectrum of [$V^{IV}O$(apoTF)] (Figure 1, II) is axial because of the slow tumbling motion of the protein. However, on the parallel part only two types of vanadium species are clearly observable (A and B), while the not completely perfect simulation of the spectrum might indicate the presence of a third species (B_2). The measured ratio of environments A and B is almost identical, but the difference between the two –7/2 peaks is only 1.5 mT (instead of 2.5 mT as reported earlier in ref.[33]).

The –3/2 peak of the perpendicular part of the LNT X-band spectra clearly shows three species in contrast with Chasteen's earlier findings, where only the Q-band spectra could differentiate between the B_1 and B_2 environments. In

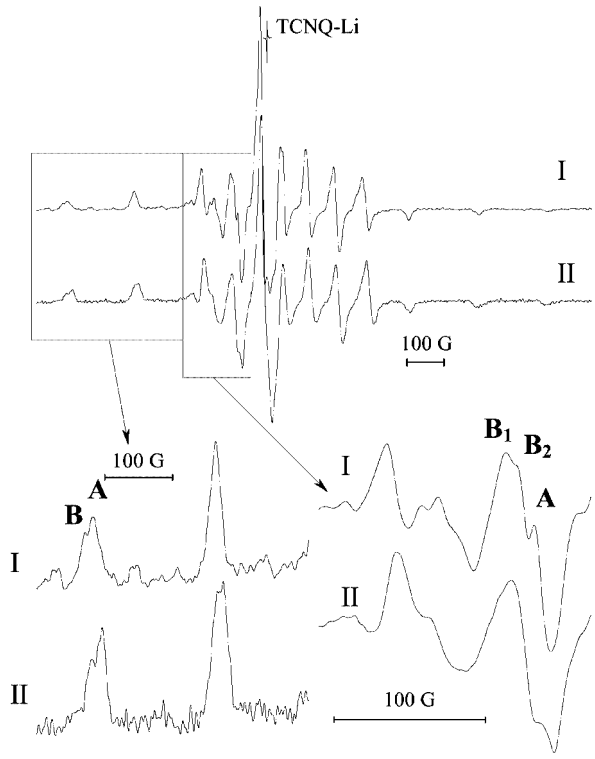
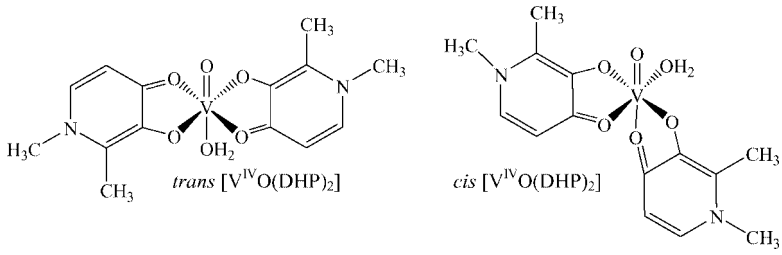


Figure 1. EPR spectra of $V^{IV}O$–apoTF, 1:1 at pH 7.5 (I) at liquid nitrogen temperature and (II) at room temperature.

the parallel part of the spectra, the peaks strongly overlap and accordingly $g_{\|}$ and $A_{\|}$ parameters were not determined.

The mechanism of the insulin-enhancing effect of $V^{IV}O$ compounds is still unknown, and the speciation of the $V^{IV}O$ insulin-mimetic compounds in the blood is therefore interesting.[34–37] The estimated [$V^{IV}O$(apoTF)] stability constants obtained from the LFER prediction clearly demonstrated that in most cases of the insulin-mimetic $V^{IV}O$ complexes (with maltol, picolinic acid, 6-Me-picolinic acid, 2-OH-pyridine *N*-oxide and 2-SH-pyridine *N*-oxide)[32,36] apoTF completely displaces the carrier ligands in equimolar concentration. Orvig et al. recently reached a similar conclusion with regard to the interactions of [$V^{IV}O(maltol)_2$] with the serum proteins albumin and apoTF:[38] TF is capable of binding two equivalents of the complex to produce a [$(V^{IV}O)_2$(TF)] species in which the metal ions occupy both Fe^{III} binding sites with the concomitant release of the free maltol. The bis complex of DHP seems to be an exception – only partial displacement of the DHP occurred because it forms exceptionally stable complexes with $V^{IV}O$.[37] Although [$V^{IV}O(DHP)_2$] is present exclusively at pH 7.5 (see speciation in Figure 2), the EPR spectrum obtained at this pH (Figure 3a: LNT, 3b: room temperature) indicates the presence of two isomers. The formation of *cis* and *trans* isomers (see Scheme 1) among the bis-$V^{IV}O$ complexes of chelating O-donor ligands is well documented in the literature.[34–37] There is no general rule regarding the preferential formation of one or other of the isomers, but it has been found, for instance, that the charge of the coordinating ligands and the size of the chelate ring can affect this.[39] The



Scheme 1. Binding modes of the *cis* and *trans* isomers of $[V^{IV}O(DHP)_2]$.

EPR parameters of the bis complexes of $V^{IV}O$ and DHP, together with the ratios of the two isomers, are given in Table 1. The data reveal that *trans* isomer formation is favoured with DHP, when a water molecule is *trans* to the oxo group of $V^{IV}O$.

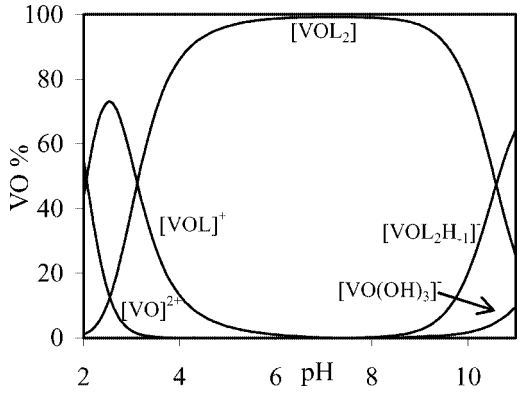


Figure 2. Speciation curves for complexes formed in the $V^{IV}O$–DHP system at a 1:2 metal-ion-to-ligand ratio, $[V^{IV}O]$ = 0.001 M. Calculated with stability constants, $\log\beta(HL)$ = 9.76, $\log\beta(H_2L)$ = 13.46, $\log\beta([V^{IV}OL]^+)$ = 12.18, $\log\beta([V^{IV}OL_2])$ = 22.83, $\log\beta([V^{IV}OL_2H_{-1}]^-)$ = 12.24, $\log\beta([V^{IV}OL_3H_2]^+)$ = 38.5, $\log\beta([V^{IV}O_2L_2H_{-2}]^{2-})$ = 16.43, taken from ref.[40]

After the detailed equilibrium and EPR spectral description of the binary systems, measurements were made on the ternary system $V^{IV}O$–apoTF–DHP at different metal-ion-to-ligand ratios at both room temperature and 77 K. For easier comparison, the room temperature spectra of the $V^{IV}O$–apoTF (1:1), $V^{IV}O$–DHP (1:2) and $V^{IV}O$–apoTF–DHP (1:1:2) systems are depicted together in Figure 4. It is clearly seen that the ternary spectrum is a composite of the isotropic spectrum of the $[V^{IV}O(DHP)_2]$ and the axial one of the $[(V^{IV}O)(apoTF)]$. This observation indicates partial displacement of the carrier ligand by apoTF or vice versa, i.e. $[V^{IV}O(DHP)_2]$ + apoTF ⇌ $[(V^{IV}O)(apoTF)]$ + 2 DHP. (As a first approximation, ternary complex formation with the protein and the carrier ligand was neglected in order to make further calculations possible.) The +7/2 and +5/2 parts of the LNT spectra of these three systems are depicted together in Figure 5.

As a first step in the quantitative evaluation of the spectral changes, the extent of ligand displacement was determined by calculating the ratio of the concentrations of $V^{IV}O$ bound to the two ligands through double integration

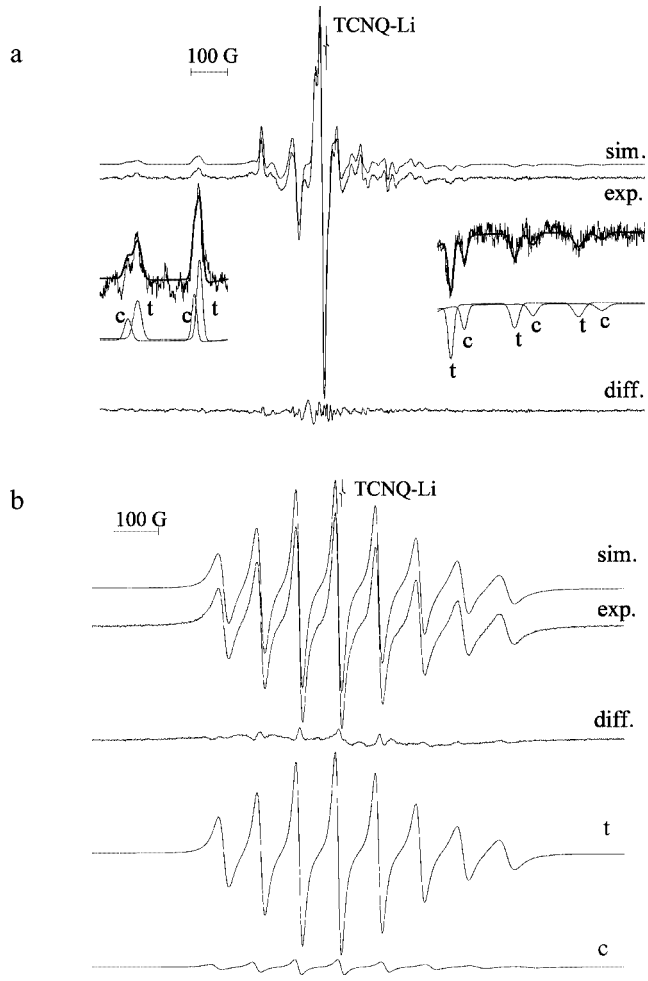


Figure 3. EPR spectra (sim. = simulated, exp. = experimental) of $V^{IV}O$–DHP, 1:2 at pH 7.5 (a) at liquid nitrogen temperature and (b) at room temperature, *t* = *trans* isomer, *c* = *cis* isomer.

of the enlarged sections of the simulated EPR spectra shown in Figure 5. From these data, the concentrations ($[V^{IV}O(apoTF)]$, [DHP], $[V^{IV}O(DHP)_2]$ and [apoTF]) and the displacement constant $K = [[V^{IV}O(apoTF)]]\cdot[[DHP]]^2/[[V^{IV}O(DHP)_2]]\cdot[[apoTF]]$ can be calculated. We used this displacement constant ($\log K = -3.6 \pm 0.3$) to obtain the binding constant for the $V^{IV}O$–apoTF interaction. The conditional stability constant for the competitive complex $[V^{IV}O(DHP)_2]$ at pH 7.5 was first calculated by taking into account the protonation equilibria of the ligand DHP, Equation (1):

Table 1. EPR parameters[a] for the $V^{IV}O$–apoTF, $V^{IV}O$–DHP and $V^{IV}O$–apoTF–DHP systems obtained by computer simulation[44] of the experimental spectra, together with some earlier literature results.

	g_x, g_y	g_z	A_x, A_y	A_z	Ratio	pH	Ref.
$V^{IV}O$–apoTF							
RT							
A	1.974(1)	1.940(2)	60.1(5)	166.8(9)		7.5	[b]
B	1.969(1)	1.934(3)	56(1)	170(3)		7.5	[b]
LN							
A	1.973(1)	1.939–1.944[d]	55.6(8)	166–171[d]		7.5	[b]
B_1	1.972(1)	1.939–1.944[d]	57.4(4)	166–171[d]		7.5	[b]
B_2	1.976(1)	1.939–1.944[d]	59.7(7)	166–171[d]		7.5	[b]
A	1.973	1.938	56.6	168.0	50	7.6	[14]
B_1	1.979	1.941	57.7	170.3	25	7.6	[14]
B_2	1.977	1.937	60.9	172.4	25	7.6	[14]
A	1.973	1.938	57.5	168.0		8.0	[27]
B	1.978	1.9375	54.0	170.0		8.0	[27]
Rat TF	1.968	1.937	57.9	167.7		8.3	[33]
$V^{IV}O$–DPH							
RT							
$V^{IV}O(L)_2$ *trans*[c]	1.973(1)		84.2(2)		88(5)		[b]
$V^{IV}O(L)_2$ *cis*[c]	1.967(2)		92(2)		12(5)		[b]
LN							
$V^{IV}O(L)_2$ *trans*	1.987(1), 1.981(1)	1.954(1)	43.2(3), 54.4(1)	158.1(2)	64(4)		[b]
$V^{IV}O(L)_2$ *cis*	1.978(1)	1.943(1)	58.1(4)	168.3(2)	36(4)		[b]
$V^{IV}O$–apoTF–DHP							
RT							
$V^{IV}O$(apoTF) A (?)	1.972(1)	1.940(1)	57.5(1)	164.9(2)		7.5	[b]
$V^{IV}O(L)_2$ *trans*[c]	1.973(1)		84.2(4)			7.5	[b]
$V^{IV}O(L)_2$ *cis*[c]	1.967(2)		92(1)			7.5	[b]

[a] In the case of the axial geometry: $g_x = g_y = g_\perp$; $A_x = A_y = A_\perp$; $g_z = g_\parallel$ and $A_z = A_\parallel$. A is given in 10^4 cm^{-1}. [b] This work. [c] Isotropic spectra $g_x = g_y = g_z = g_0$ and $A_x = A_y = A_z = A_0$. [d] The parameters are not resolved.

$$\log \beta^c([V^{IV}O(DHP)_2]) = \log \{\beta([V^{IV}O(DHP)_2])/(1 + \beta_1^{II}(DHP)\ 10^{-pH} + \beta_2^{II}(DHP)\ 10^{-2pH})^2\} \quad (1)$$
$$= \log (10^{22.83}/(1 + 10^{9.76}10^{-7.5} + 10^{13.46}10^{-15.0})^2 = 18.31$$

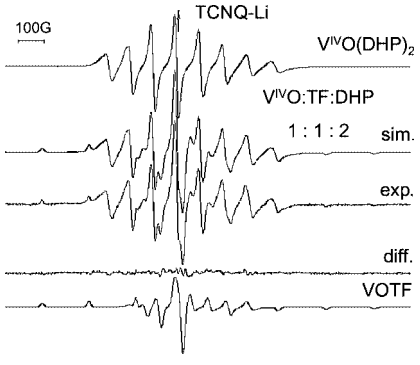


Figure 4. Room-temperature EPR spectra of (a) the binary $V^{IV}O$–DHP 1:2 (simulation), (b) the ternary $V^{IV}O$–apoTF–DHP 1:1:2 and (c) the $V^{IV}O$–apoTF 1:1 systems (simulation) at pH 7.5. Simulations were performed with EPR parameters given in Table 1.

A value of $\log\beta^c([V^{IV}O(DHP)_2]) = 18.31 \pm 0.06$ was obtained. (Stability constants for the formation of the proton and $V^{IV}O$ complexes were taken from ref.[40]) The conditional binding constant could now be calculated as $\log K_1^c([V^{IV}O(apoTF)]) = \log\beta^c([V^{IV}O(DHP)_2]) + \log K_{displacement} = 18.31 - 3.6 = 14.7$. The ratios ($[V^{IV}O(apoTF)]/[V^{IV}O(DHP)_2]$) and the $\log K_1^c([V^{IV}O(apoTF)])$ values determined for all spectra are listed in Table 2. From EPR spectroscopic studies, where formation of both the ternary complex and $[(V^{IV}O)_2(apoTF)]$ was neglected, $K_1^c([V^{IV}O(apoTF)]) = 14.7 \pm 0.3$ was determined. The binding constant $\log K_2^c$, characteristic of the binding of the second $V^{IV}O$ to apoTF, can be estimated by using the observed difference between the stepwise binding constants for transition metal ions (ca. one log unit),[2] as $\log K_2^c([V^{IV}O(apoTF)]) = 13.7 \pm 0.5$.

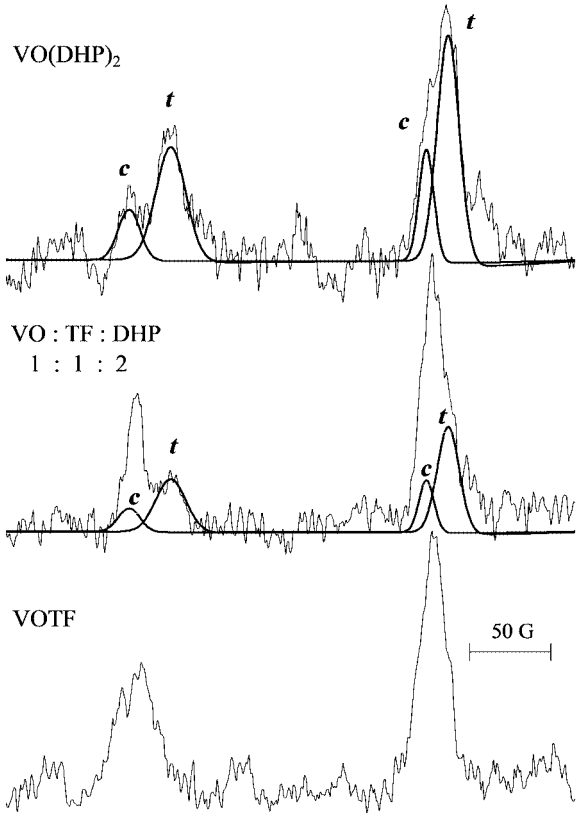


Figure 5. Lowfield region (parallel +7/2, +5/2 peaks) LNT EPR spectra of (a) the binary $V^{IV}O$–DHP 1:2, (b) the ternary $V^{IV}O$–apoTF–DHP 1:1:2 and (c) the $V^{IV}O$–apoTF 1:1 systems at pH 7.5.

In order to confirm the conditional [$V^{IV}O$(apoTF)] binding constants obtained, UV spectrophotometry was also used in the hope that this would furnish more accurate binding constants. In this case, we had to apply a rather low apoTF concentration (8 μM) so as to be able to measure the UV absorbance, and we used a different second ligand, NTA, to displace apoTF from the vanadium coordination sphere. The shape and the intensity of the individual difference spectra (see Figure 6) are in good agreement with that reported by Bertini et al. earlier.[20]

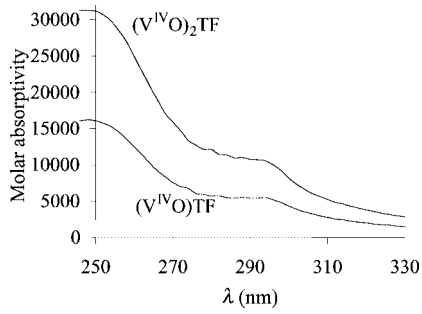


Figure 6. Individual difference spectra of [$V^{IV}O$(apoTF)] and [$(V^{IV}O)_2$(apoTF)] calculated from UV spectroscopic measurements.

The determined $\log K_1^c$ and $\log K_2^c$ values are listed in Table 2. Unfortunately, there is a 0.7 unit difference in the $\log K_1^c$ values determined with DHP and NTA, and both are outside the uncertainty range of the same constants determined by EPR spectroscopy.

Table 2. Stepwise stability constants {$V^{IV}O^{2+}$ + apoTF ⇌ [$V^{IV}O$(apoTF)] ($\log K_1^c$) and VO^{2+} + [$V^{IV}O$(apoTF)] ⇌ [$(V^{IV}O)_2$(apoTF)]} ($\log K_2^c$) determined by different methods {pH 7.5, [HCO_3^-] = 0.025 M, T = 25 °C}.

Method	System	[$V^{IV}O$(apoTF)]: [$V^{IV}O(DHP)_2$]	$\log K_1^c$		$\log K_2^c$		Numb. of spectra	Fitting[a]
LNT EPR	$V^{IV}O$–apoTF–DHP (mM)							
	0.5 : 0.5: 1.0	40 : 60	15.0	±0.3			1	
	0.5 : 0.75 : 1.5	25 : 75	14.6	±0.3			1	
RT EPR	$V^{IV}O$–apoTF–DHP (mM)				13.7[b]	±0.5		
	1 : 1 : 2	37 : 63	14.9	±0.1			1	
	1 : 0.5 : 1	47 : 53	14.6	±0.1			1	
	1 : 1 : 1	59 : 41	14.3	±0.2			1	
UV–VIS								
	$V^{IV}O$–apoTF–DHP		14.0	±0.2	11.5	±0.6	70	5.9×10^{-3}
	$V^{IV}O$–apoTF–NTA		13.4	±0.2	11.9	±0.5	85	3.9×10^{-3}
	$V^{IV}O$–apoTF–DHP–NTA		13.7	±0.2	11.8	±0.5	129	5.7×10^{-3}
Average			**14.3**	**±0.6**	**11.7**	**±0.6**		

[a] Square root of the average of the square difference between the measured and simulated absorbance; sqrt{$\Sigma[(A_{exp} - A_{fitt})^2]/n$}, n is the number of degrees of freedom. [b] Estimated value from ref.[2] (see text).

The difference between the binding constants determined from the EPR and UV spectra may be explained in part by the slightly different experimental conditions: different total concentrations of apoTF, ca. 1 mM for EPR and ca. 0.01 mM for UV spectroscopic measurements; different solvents, 10% (v/v) DMSO for the LNT EPR spectroscopy and water for the other measurements; and different temperatures, ca. –5 °C for the LNT measurements and 25 °C for the others.

The rather low value obtained for $\log K_2^c$ by UV spectroscopy is more worrying. However, this value is probably very uncertain because of the very low concentration of the ligand used in the UV spectroscopic measurements, and thus the rather high error from any disturbing circumstances affecting the concentration of the interacting species, or from the light absorption of the sample, such as slight oxidation of the metal ion. Accordingly, $\log K_2^c$ determined by means of UV spectroscopic measurements can be considered only a tentative value. The constants K_1^c and K_2^c are conditional constants for pH 7.5 and 25 mM hydrogen carbonate concentration.

The differences between the binding constants $\log K_1^c$ obtained by the three experimental methods (room-temperature EPR spectroscopy, LNT EPR and UV spectroscopy) are about 0.5–1.5 orders of magnitude higher than that obtained from the LFER prediction (13.2 ± 1.6) (see Figure 6 in ref.[32]), although they are within the uncertainty range of the latter.

As mentioned above, the absence of ternary complex formation between [$V^{IV}O$(apoTF)] and the competitor ligand had to be assumed as a precondition for the calculation of the binding constant. This has not been proven,[38] and NMRD measurements[41] have suggested that displaced DHP ligand(s) do not move far away from the metal ions, which to some extent continues to feel their presence. More detailed studies are currently being carried out in our laboratories in order to explore the possibility of ternary complex formation between $V^{IV}O$–apoTF and low molecular mass carrier ligands. This process may be the reason for the differing LFER estimated value.

Although the measurements reported here were carried out with metal-ion-free apoTF, the binding constants determined can be applied with a good approximation to the interaction of $V^{IV}O$ with TF, as only ca. 30% of the metal-binding sites of the protein are saturated with Fe^{III} under normal conditions, and thus there are enough free sites to bind other, mostly hard metal ions (e.g. Al^{III} or $V^{IV}O$) and to transport them in the blood stream.

Materials and Methods

Chemicals

DHP, NTA and human apotransferrin (apoTF) were obtained from Sigma Co. The purities of the small organic ligands were checked, and the exact concentrations of their solutions were determined by the Gran method.[42] The concentrations of protein solutions were estimated from their UV absorption (ε_{280}(TF) = 92.300 $m^{-1} cm^{-1}$[24]). The $V^{IV}O$ stock solution was prepared as described in ref.[43] and standardised for metal ion concentration by a permanganate titration.

EPR Spectroscopic Measurements

As a consequence of the ready oxidation of $V^{IV}O$ to V^{V} under neutral or basic conditions, extreme care was taken to exclude oxygen during the preparation of the samples and during the measurements. It has been reported that $V^{IV}O$ ions bound to human serum TF are very unstable towards aerobic oxidation,[24] and the half-life for the oxidation is estimated to be between 5 and 13 min. Aqueous solutions (0.5 mL) of samples containing DHP and apoTF in ratios of 0.001 M:0.0005 M, 0.0015 M:0.0005 M and 0.001 M:0.0008 M were prepared in 0.1 M HEPES buffer and 0.025 M sodium hydrogen carbonate adjusted to pH 7.5. The solutions were carefully purged with argon and then sealed under an argon atmosphere. A $V^{IV}O$ stock solution (5 μL) was then added to each sample through a rubber septum with a Hamilton syringe. Samples for the EPR spectroscopic measurements were taken out by syringe and were immediately transferred into the EPR tube (LNT) or into the capillary (room temperature). X-band EPR spectra (9.59 or 9.40 GHz) were recorded in aqueous solution at LNT (77 K) and at room temperature with a Bruker EMX or JEOL JES-RE-1X spectrometer. The samples for low-temperature measurements contained 10% (v/v) of DMSO to ensure good glass formation (dilution of the samples was taken into account). The EPR spectra were evaluated and simulated with the EPR computer programme of Rockenbauer and Korecz.[44]

UV Spectroscopic Measurements

At physiological pH, vanadium(IV) undergoes extensive hydrolysis. Hence, the stability constants of apoTF were determined by a displacement reaction with NTA and with DHP. Both ligands form appropriately stable complexes with $V^{IV}O$, and the stability constants are available at 25 °C and I = 0.2 M (KCl).[40,45] However, while NTA does not exhibit significant UV absorption at pH 7.5, which simplifies the determination, at pH 7.5 the complex $[V^{IV}OL]^-$ of NTA is partly hydrolysed and a ternary complex, $[V^{IV}OL(OH)]^{2-}$, is formed, which makes the system somewhat more complicated. On the other hand, DHP forms a single $[V^{IV}OL_2]$ complex at pH 7.5, but both the complex and the ligand exhibit significant UV absorption, which imposes a limit to the use of a high excess of the ligand.

In contrast with the difference UV spectroscopic method (frequently applied by Harris and coworkers to determine the stability constants of TF complexes with different metal ions[4–9]), we simply measured the absorbance and fitted the data (wavelength range 250–330 nm) and individual spectra by using the computer programme PSEQUAD.[46]

The apoTF concentration was 8 μM, the molar ratio between apoTF and NTA was 1:0, 0:1, 1:1, 1:5, 1:25 or 1:120 and that between apoTF and DHP was 0:1, 1:1, 1:2.5 or 1:5 at 25 °C in 0.025 M HCO_3^-, and I = 0.2 M (KCl) in 0.1 M HEPES buffer adjusted to pH 7.5. The starting volume was 2.0 mL and at each titration point 25 μL of 0.16 mM $V^{IV}OCl_2$ was added until an apoTF to $V^{IV}O$ ratio of 1:4 was reached. (When the calculation suggested hydrolysis of $V^{IV}O$ at a titration point, the data of the spectrum were omitted from the calculation.) An HP 8452 diode array spectrophotometer was used with a silica cell with a pathlength of 1.00 cm. During measurements, the cell was closed, and during the addition of $V^{IV}O$, argon was bubbled through the sample to exclude air.

Acknowledgments

This work was performed in the frame of a COST D21 collaboration and supported by the Hungarian Research Fund (OTKA grants NI61786 and T049417). The authors thank Dr E. A. Aad (Universite du Littoral) for his help with the EPR spectroscopic measurements.

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Received: April 6, 2006
Published Online: August 15, 2006

