The Oxyvanadium Constellation in Transition-State-Analogue Complexes of Phosphoglucomutase and Ribonuclease. Structural Deductions from Electron-Transfer Spectra†

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ABSTRACT: The absorbance peak in the near ultraviolet electron-transfer spectrum of the oxyvanadium constellation in the "transition-state-analogue complexes" obtained by treating the dephospho form of phosphoglucomutase with inorganic vanadate in the presence of either glucose 1-phosphate or glucose 6-phosphate, as described in an accompanying paper [Ray, W. J., Jr., Burgner, J. W., II, & Post, C. B. (1990) *Biochemistry* (second of four papers in this issue)], is centered at a wavelength of 312 nm. The position of this peak amounts to a change in oscillator frequency of about -5000 cm^{-1} relative to that of tetrahedral VO_4^{3-}. To provide a rationale for this spectral change, the near ultraviolet spectra of the *di*- and *mono*anions of inorganic vanadate and a number of derivatives of these anions are compared with that of vanadium(V) in the enzymic complexes, in terms of both what is observed experimentally and what is expected from crystal field theory. Comparisons in water and in largely anhydrous solvents show that water is not an essential element in the coordination sphere of inorganic vanadate or its *mono*- or *di*esters and hence that the coordination number of V(V) in such compounds likely is four. These comparisons also show that loss of solvating water from a 4-coordinate vanadate on binding cannot provide a rationale for the spectra of the enzymic complexes. Other comparisons show that neither the binding of metal ions nor protonation nor the binding of vanadate at a site with an unusually high or an unusually low dielectric constant can provide such a rationale. Further comparisons with vanadates known to be pentacoordinate strongly suggest that the coordination number of V(V) in the transition-state-analogue complexes of phosphoglucomutase does not exceed four. In fact, from the standpoint of crystal field theory the marked red shift observed in the electron-transfer absorbance spectrum of the oxyvanadium constellation in these complexes is more reasonably interpreted in terms of a *decreased* coordination at vanadium(V), viz., in terms of a weakened bonding between vanadium and one or more of its coordinating oxygens. This decreased coordination could be produced by a physical stretching of the vanadate ester linkage. By contrast, the near ultraviolet spectrum of the transition-state-analogue complex that ribonuclease forms with an adduct of uridine and vanadate [Lindquist, R. N., Lynn, J. L., & Lienhard, G. E. (1973) *J. Am. Chem. Soc. 95*, 8762] is similar to spectra of pentacoordinate model compounds of vanadium(V). An assessment of stability for the ribonuclease/uridine/vanadate adduct by comparison with the vanadate adduct of β-methyl riboside is described. The results suggest that this adduct binds more tightly to ribonuclease than has been thought. If the extent of chemical bonding in these vanadate complexes reflects the structure of the transition states in the respective reactions catalyzed by phosphoglucomutase and ribonuclease, those transition states likely are characterized by a significantly higher degree of bond *breaking* than bond making in the case of phosphoglucomutase and by a significantly higher degree of bond *making* than bond breaking in the case of ribonuclease.

The previous paper in this series (Ray et al., 1990) describes studies which show that the mixed phosphate/vanadate diesters of glucose, V-6-Glc-1-P and V-1-Glc-6-P,[1] bind to the dephospho form of phosphoglucomutase with the vanadate ester grouping at the (PO_3^-)-transfer site[2] (the proximal phosphate site) and the phosphate ester grouping at the phosphate-binding site (the distal site) (see Scheme I of the above paper). Because of both this binding pattern and the greatly increased affinity of the dephospho enzyme for the mixed phosphate/vanadate diester, relative to the bisphosphate ester that normally binds to the dephospho enzyme [cf. the fourth paper in this series (Ray & Puvathingal, 1990)], it seems likely that chemical bonding within the bound oxyvanadium constellation provides a reasonably accurate reflection of bonding in the

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[1] Abbreviations: E-P and E, the phospho and dephospho forms of phosphoglucomutase; Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, D-glucose 6-phosphate—equilibrium mixture of α and β anomers unless otherwise specified; Glc-P_2, and P-6-Glc-1-P, or P-1-Glc-6-P, α-D-glucose 1,6-bisphosphate; V_i, inorganic vanadate, V-6-Glc-1-P and V-1-Glc-6-P, the 6-vanadate ester of Glc-1-P and the α-1-vanadate ester of Glc-6-P; E*V*6-Glc-1-P and E*V*1-Glc-6-P, the inhibitor complexes produced by treating the Mg^{2+} form of the dephospho enzyme with V_i plus Glc-1-P or Glc-6-P, respectively; E-V-6-Glc-1-P(Li^+), the corresponding complex produced with the Li^+ form of the enzyme; DMSO, dimethyl sulfoxide; CHES, (*N*-cyclohexyl-2-amino)ethanesulfonic acid; CAPS, (*N*-cyclohexyl-3-amino)propanesulfonic acid; MES, morpholinoethanesulfonic acid.

[2] "(PO_3^-) transfer" is used herein to designate the process $R_1OPO_3^{2-} + R_2OH \rightarrow R_1OH + R_2OPO_3^{2-}$ and refers to the identity of the group transferred, without implication about the mechanism of transfer.

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transition state for the (PO_3^-)-transfer process catalyzed by phosphoglucomutase. In such a case one might deduce some of the properties of that transition state by making judicious comparisons with model compounds. Of particular importance is whether there is independent evidence that the hydroxyl group of the active-site serine residue of the enzyme adds to the bound vanadate ester to give a pentacoordinate adduct, as is suggested by Percival et al. (1990).

Our approach takes advantage of the fact that vanadate(V) and its derivatives, which frequently are used as phosphate analogues, exhibit moderately intense electron-transfer spectra. However, a basic problem in attempting to answer structural questions about vanadates via spectroscopy is the paucity of such compounds whose structure is known [in contrast with complexes of V(III) and V(IV); cf. Clarke (1976)] and the difficulty of establishing conditions in solutions of V(V) where a single species is present. For example, in aqueous alcohol at slightly basic pH a solution will contain both *mono-* and *di*anionic forms of inorganic vanadate, $(HO)_2VO_2^-$ and $HOVO_3^{2-}$, plus *mono-* and *di*anionic forms of the monoester, $(RO)(HO)VO_2^-$ and $ROVO_3^-$, respectively, as well as the diester of the *mono*anion, $(RO)_2VO_2^-$. In addition, unlike phosphates, vanadate esters come to thermodynamic equilibrium with hydroxyl compounds within seconds, so that a unique vanadate cannot be prepared externally and examined as such in aqueous solution. Fortunately, recent studies by Gresser and co-workers and by Tracey and co-workers have provided sufficient data on structure and equilibrium constants that it is now possible to establish conditions for a number of vanadates where either a single species heavily predominates or where the composition of the mixture is known with sufficient accuracy to resolve the spectrum of the mixture into its component spectra, at least approximately.

On the basis of both spectral comparisons and theory, a pentacoordinate adduct does not appear to be a viable possibility for the structure of the transition-state-analogue complexes of phosphoglucomutase. Hence, after considering reasonable alternatives, we refer to the analogue complexes derived from Glc-1-P and Glc-6-P, respectively, as E*V*6-Glc-1-P and E*V*1-Glc-6-P. This convention emphasizes their respective structural relationship to the normal enzymic complexes, E-P-Glc-1-P or E-P-6-Glc-1-P, where the 1-phosphate is at the distal site [see Scheme I of the second paper in this series (Ray et al., 1989)], and to E-P-Glc-6-P or E-P-1-Glc-6-P, where the 6-phosphate is at the distal site. This convention also is intended to convey a degree of uncertainty about the actual bonding within the oxyvanadium constellations of these complexes. A thermodynamic comparison of the binding of V-6-Glc-1-P and P-6-Glc-1-P to the dephospho enzyme is described in the fourth paper of this series (Ray & Puvathingal, 1990).

Conclusions that can be drawn about the transition-state-analogue complex that ribonuclease forms with uridine and vanadate also are considered on the basis of previously published work and spectral studies described herein. These studies include an evaluation of the stability of the uridine/vanadate complex by comparison with the analogous complex involving β-methyl riboside.

Experimental Procedures

Materials. Imidazole (Aldrich) was treated as described in the accompanying paper and brought to pH 7 before use. CAPS, CHES, and MES buffers (CalBiochem) were used without further purification. A 0.5 M stock solution of inorganic vanadate was obtained by dissolving V_2O_5 (Alfa) in an aqueous solution that contained 2.2 equiv of KOH or 4 equiv of tetramethylguanidine (Eastman). Where possible, spectrograde ligands were used to form complexes with inorganic vanadate; otherwise, reagent grade ligands were used without further purification. Aqueous solutions of butane-2,3-diol (40%) and dioxane (95%) were treated to free them of peroxides via procedure b in Ray and Puvathingal (1985). 18-Crown ether (Eastman) was dissolved in about 5 volumes of water and maintained at pH 11 for several hours (during which time about 3 mol % base was consumed). After the solution stood overnight and the pH was readjusted to 11, the ether (plus generated salt) was isolated by lyophilization. A 0.36 M solution of β-methyl riboside (Sigma) was passed slowly through a 0.6 × 4 cm column of activated charcoal and then through a 0.4-μm filter before use. Solutions of *cis-* and *trans*-cyclohexanediol (K and K and Aldrich, respectively) were treated with large amounts of charcoal before use.

Procedures. Most spectra of vanadate and various adducts (at 25 °C) were recorded after 5—10 μL of inorganic vanadate, 1–5 mM, was added to 1.0 mL of sample and 5–10 μL of water was added to 1.0 mL of the reference solution, both of which contained the ligand to be tested. Teflon-stoppered sample and reference cells were used in a Perkin-Elmer Lambda 6 spectrophotometer. (Many of the solutions employed exhibited a significant absorbance in the near ultraviolet region; hence, a difference technique was employed to minimize the effect of diluting these solutions by the addition of vanadate.) In largely aqueous solutions, pH values were adjusted with a meter. Spectra were recorded at 120 nm/min with a 4-mm slit width and a response setting of 2. When the final vanadate concentration was less than 25 μM, multiple scans of both base line and spectra were obtained and averaged. Base lines were subtracted digitally with the Perkin-Elmer PECSS program. In alcoholic solutions, an effective pH of 7.0 or 11.0 was maintained by use of 20 mM buffer, imidazole or CAPS, respectively, when the concentration of alcohol was 50% or less. The spectrum of the complex that ribonuclease forms with uridine and vanadate was obtained via a one-cell procedure which utilized an absorption cell equipped with a quartz insert (Helma) that reduced the light path of the solution to 0.0052 cm. Spectra of identical solutions with and without vanadate were recorded successively and subtracted, digitally.

The published formation constants identified below were used under the indicated conditions to calculate the concentrations of vanadate complexes present in most of the solutions whose spectra appear in Figure 1. Formation constants are given relative to $[H_2O]$ = 55 M; alcohol concentrations are given as the mole ratio, *R*, with respect to water (as well as the v/v percent, in parentheses). When published data [see Tracey et al. (1988a) and references cited therein] indicated that more than one complex was present, spectra of the mixtures at two or more concentrations were resolved into molar absorbances of the components by algebraic manipulation of spectra with the Perkin-Elmer PECSS program, according to published constants. (For assumptions involved, see Results.) Unless otherwise indicated, spectra were obtained at 50 μM total vanadate, although in representative cases spectra were checked at 10 μM to verify the absence of polymeric species.[3] For methanol/water solutions, formation constants were 5.2 M^{-1} (monoanion monoester), 6.2 M^{-2} (monoanion diester), and 3.3 M^{-1} (dianion ester). The pH, the methanol concentrations used, and the calculated fractional distribution of vanadium

[3] The concentration independence of apparent molar extinctions was taken as evidence for the absence of polymeric species (see footnote *c*, Table I).

were, at pH 7 and R = 0.07 (13.5%), $H_2VO_4^-$ = 0.72, $CH_3OVO_3H^-$ = 0.26, and $(CH_3O)_2VO_2^-$ = 0.022; at pH 7 and R = 0.35 (44%), $H_2VO_4^-$ = 0.28, $CH_3OVO_3H^-$ = 0.51, and $(CH_3O)_2VO_2^-$ = 0.22; and at pH 11 and R = 0.234 (33%), HVO_4^{2-} = 0.56 and $CH_3OVO_3^{2-}$ = 0.44. For ethanol/water solutions, formation constants were 11.6 M^{-1} (monoanion monoester), 25.9 M^{-2} (monoanion diester), and 2.78 M^{-1} (dianion ester). The ethanol concentrations used and the calculated fractional distribution of vanadium were, at pH 7 and R = 0.031 (10%), $H_2VO_4^-$ = 0.73, $C_2H_5OVO_3H^-$ = 0.25, and $(C_2H_5O)_2VO_2^-$ = 0.019; at pH 7 and R = 0.324 (40%), $H_2VO_4^-$ = 0.13, $C_2H_5OVO_3^-$ = 0.48, and $(C_2H_5O)_2VO_2^-$ = 0.38; and at pH 11 and R = 0.10 (30%), $C_2H_5OVO_3^{2-}$ = 0.28 and $HOVO_3^{2-}$ = 0.72. For trifluoroethanol/water solution, formation constants were 3.9 M^{-1} (monoanion monoester and dianion ester) and ≈0 M^{-2} (monoanion diester). The concentrations used and the calculated fractional distribution of vanadium at pH 7 were, at R = 0.106 (30%), $(HO)_2VO_3^-$ = 0.69, $CF_3CH_2OVO_3H^-$ = 0.28, and $(CF_3CH_2O)_2VO_2^-$ = 0.038; at R = 0.246 (50%), $(HO)_2VO_2^-$ = 0.45, $CF_3CH_2OVO_3^-$ = 0.43, and $(CF_3CH_2O)_2VO_2^-$ = 0.12; and at 0.01–0.05 N KOH and R = 0.11 (34%); $HOVO_3^{2-}$ = 0.67 and $CF_3CH_2OVO_3^{2-}$ = 0.33. For 2-propanol/water solutions, formation constants were 13.9 M^{-1} (monoanion monoester), ≈0 M^{-2} (monoanion diester), and 0.014 M^{-1} (dianion ester). The concentrations used and the fractional distribution of vanadium at pH 7 were, at R = 0.10 (30%), $(HO)_2VO_2^-$ = 0.42 and i-$PrOVO_3^{2-}$ = 0.58 and, at 1 mM KOH and R = 0.24 (50%); $HOVO_3^{2-}$ = 0.81 and i-$PrOVO_3^{2-}$ = 0.18. For hexafluoro-2-propanol/water solutions, formation constants were 3.3 M^{-1} (monoanion monoester) and ≈0 M^{-2} (monoanion diester). The concentration used and the calculated fractional conversion of $(HO)_2VO_2^-$ to the monoester monoanion at pH 7 and R = 0.073 (30%) was 0.23. For *tert*-butyl alcohol/water solutions, formation constants were 5.3 M (monoanion monoester) and ≈0 M^{-2} (monoanion diester); the *tert*-butyl alcohol concentrations used and the calculated fraction of V_i present as t-$BuOVO_3H^-$ at pH 7 were, at R = 0.037 (16.3%), 0.126; at R = 0.056 (22.6%), 0.178; and at R = 0.074 (28%), 0.218.

Titration of β-Methyl Riboside with Vanadate(V). A solution, 5.00 mL, 1.2 M in β-methyl riboside, and 20 mM in imidazole, pH 7, was titrated by addition of 4 μL from each of a series of stock solutions of vanadate. [β-Methyl riboside concentration was estimated by the periodate procedure (Dryhurst, 1970)]. Before the first addition, and subsequent to each addition thereafter, a portion of the solution (about 4 mL) was transferred to a cylindrical, quartz absorption cell (Helma) with a light path of 5 cm that was firmly fixed in the sample beam of the Lambda 6 spectrophotometer. After the spectrum was recorded, the sample was returned to the original container. Multiple transfer steps were employed after each addition of vanadate to ensure mixing. At the lower V_i concentrations, multiple scans (up to eight) were made before and after the addition and later averaged. At the higher concentrations, only two scans were used.

NMR Studies. ^{31}P NMR spectra were accumulated with an XL-200A spectrometer in the manner described previously (Post et al., 1989). ^{51}V NMR studies were obtained in a similar fashion except that a 16-mm probe was used, the spectrometer was operated in the unlocked mode, and chemical shifts are reported in parts per million from $VOCl_3$. A pulse angle of 90° and a recycle time of 0.1 s were used.

Results

Molar extinction spectra in aqueous solution are shown in Figure 1a for the ionic forms of inorganic vanadate, VO_4^{3-}, $HOVO_3^{2-}$, and $(HO)_2VO_2^-$, along with that of the oxyvanadium chromophore in the transition-state-analogue complexes of phosphoglucomutase, which for convenience are referred to as E*V*6-Glc-1-P, although both this complex and the E*V*1-Glc-6-P complex are present at equilibrium [cf. Ray et al. (1990)]. Of the ionic forms of V_i, only VO_4^{3-} is known with certainty to be tetrahedral (Griffith & Wickins, 1966). Hence, this species serves as a reference. To facilitate deductions about what type of changes in chemical bonding within VO_4^{3-} would be required to produce an absorbance spectrum like that of the oxyvanadium chromophore in E*V*6-Glc-1-P, the spectra of a number of vanadate esters were obtained. While it is dianionic vanadate in the mixed diester of glucose that binds to phosphoglucomutase (see Discussion), *mono*anionic vanadates also were studied to provide both correlations and contrasts with the *di*anionic species.

Identification of Species Present in Spectral Studies. When more than one species of vanadate were present at significant concentrations, as deduced from published formation constants (see Experimental Procedures), spectra of mixtures were recorded at two or more concentrations of a critical component and manipulated appropriately to obtain molar extinction spectra for the predominant species present in the mixture. (The calculated distributions of vanadate species in the solutions employed are given under Experimental Procedures.) Concentrations of 50 μM or less were used to minimize the tendency of vanadate to form oligomers (Kepert, 1973), and many molar extinctions also were checked at a concentration of 5–10 μM.[3]

Because pK_a values of vanadate are altered substantially in largely nonaqueous solvent systems, acid/base titrations usally were conducted to determine the identity of the ionic species present in such solutions, since $A_{trianion} > A_{dianion} > A_{monoanion}$ (see Figure 1a), where A represents molar absorbance. [In water, there is a broad plateau region where $HOVO_3^{2-}$ is present almost exclusively because of a difference somewhat larger than 4 in the values of pK_{a2} and pK_{a3} for vanadate (Kepert, 1973); however, that plateau is considerably narrowed in less polar nonaqueous solutions.[4]] ^{51}V NMR spectra also were used in identifying the species present in such solutions. Tetramethylguanidinium salts of vanadate were used when possible to minimize oligomerization. The potassium salt plus an excess of 18-crown-6 ether was used in solvents where tetramethylguanidine was not sufficiently basic to produce dianionic vanadate and KOH was required, instead. (The pK_a value of the conjugate acid of tetramethylguanidine, which is quite high in water, is sharply reduced in many solvent systems with a low water content, while pK_{a2} for vanadate increases markedly in such systems, so that in such solutions the monoanion may be present almost exclusively in the presence of 40 mM tetramethylguanidine.) The conditions used for the examination as well as the basis for identification of the ionic form present in a number of such solutions are summarized in Table I.

Spectra of Inorganic Vanadate(V) in Largely Nonaqueus Solutions. Figure 1b shows the spectra of the potassium salts of vanadate in two largely nonaqueous solutions, 98% DMSO and 94% dioxane, in the presence of 18-crown-6 ether. The spectrum in dioxane was obtained at 5–10 μM V_i where there

[4] In several largely nonaqueous solvent systems containing V_i, a sharp increase in optical density with increasing concentration of KOH (usually in the presence of 18-crown-6 ether) occurred close to the solubility limit of KOH, perhaps due to ion pairing.

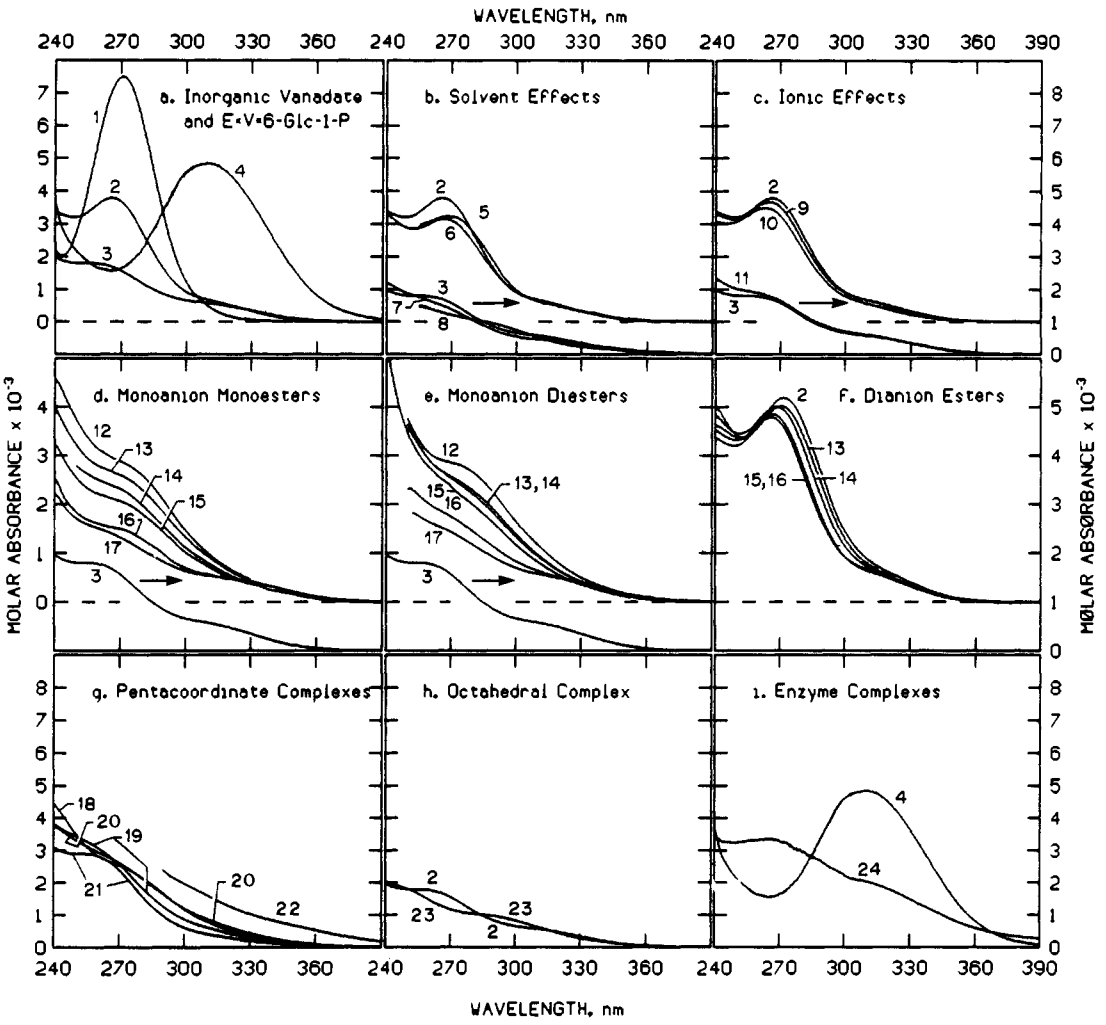


FIGURE 1: Molar absorbance spectra of inorganic vanadate and various derivatives. Unless otherwise indicated, reported spectra were obtained at 50 μM total vanadate by the procedure described under Experimental Procedures. In most cases, the pH was maintained by use of 20 mM buffers: pH 7, imidazole; pH 11, CAPS; otherwise, the pH meter reading is given. Formation constants used in resolving the spectra of mixtures are given under Experimental Procedures along with references and some conditions. Other conditions are reported in Table I. In panels b–e, the lower plots are displaced downward for clarity and the right-ordinate labels apply. (a) Spectra (left scale) of monomeric inorganic vanadate in aqueous solution: 1, VO_4^{3-}; 2, HVO_4^{2-}; 3, $H_2VO_4^-$; and 4, the oxyvanadium constellation in the E*V*6-Glc-1-P complex [from Figure 2b of Ray et al. (1990) after smoothing]. (b) Spectra of $HOVO_3^{2-}$ (upper plots, left scale) and $(HO)_2VO_2^-$ (lower plots, right scale) in organic solvents that contain only low concentrations of water. Spectra in water are given for reference. Upper plots: $HOVO_3^{2-}$ in water, 2; 97% dimethyl sulfoxide, 5; and 94% dioxane, 6. Lower plots: $(HO)_2VO_2^-$ in water, 3; 94% dioxane, 7; and 97% dimethyl sulfoxide, 8. (c) Spectra of $HOVO_3^{2-}$ (upper plots, left scale) and $(HO)_2VO_2^-$ (lower plots, right scale) in solutions of high ionic strength; spectra in water, 2 and 3, respectively, are given for reference. The spectrum of the *di*anion in 8.4 M guanidinium chloride, 9, was obtained at pH 8.6; that of the *mono*anion, 11, was obtained by extraplating the results of a spectral titration, between pH 8.6 and 6.0, to pH 5 (see Results). The spectrum of the *di*anion in 4 M $CaCl_2$, 10, was obtained at pH 8. (d) Spectra of monoanion monoesters of vanadate. Spectra of the monoesters were obtained in aqueous alcohol, pH 7 (upper spectra, left scale), by resolution of the spectra of three-component mixtures into their constituent parts: see Results and Experimental Procedures. The following alcohols were used: 12, 2-methyl-2-propanol; 13, 2-propanol; 14, ethanol; 15, methanol; 16, trifluoroethanol; and 17, hexafluoro-2-propanol. The spectrum of the monoanion in water, 3 (right scale), is shown for comparison. (e) Spectra of diester monoanions in the respective anhydrous alcohols (>99%) (left scale). *Tetra*methyl guanidine at a concentration of 20–40 mM also was present, and the di-*tetra*methylguanidine salt of V_i was used. The same alcohols as in (d) were used; these are identified as in that panel. The spectrum of the monoanion in water, 3, is shown for comparison (right scale). (f) Spectra of dianion esters in aqueous solutions of alcohols. Spectra of two-component mixtures were resolved as in (d). Four of the six alcohols employed in (c) and (d) were used and are identified in the same way. Spectrum 2 is that of the *di*anion in water. (g) Spectra of pentacoordinate complexes of vanadate. Dinuclear complexes (see c, Chart I) with 2,3-butanediol, 18, and with *cis* and *trans*-cyclohexane-1,2-diol, 19 and 20, respectively, were obtained at pH 7 in the presence of 3.5 M cyclohexanediol or 2 M 2,3-butanediol. The spectrum of the analogous complex of β-methyl riboside, 21, is the limiting spectrum obtained in the titration shown in Figure 2 at high V_i. The spectrum of V(V) in the RNase/uridine/vanadate complex (290–350 nm), 22, was obtained as the spectral difference between solutions that contained 10 mM uridine, 1.5 mM RNAse, 100 mM imidazole, pH 7, and either 0.5 or 1 mM inorganic vanadate. The essentially identical results obtained in a spectrophotometer cell with a light path of 0.0052 cm were averaged. (h) Octahedral complex of vanadate. The spectrum of the bispyrophosphate anhydride adduct (20 mM pyrophosphate, pH 7.0) is shown, 23, together with the spectrum of the monoanion in water, 3. (i) Comparison of spectra of V(V) in complexes of the enzyme involving Mg^{2+} and Li^{1+}. Spectrum 24 is that of V(V) in the E•V-6-Glc-1-P(Li^+) complex from the second paper in this series (Ray et al., 1990); spectrum 4 is that of V(V) in the E*V*6-Glc-1-P(Mg^{2+}) complex from (a).

was little concentration dependency in the apparent molar extinction. In both solvent systems the ^{51}V NMR resonance for both the *mono-* and *di*anions occurred at chemical shifts similar to those observed in aqueous solution (Table I). However, in dioxane an upfield resonance also was observed for the *mono*anion, presumably due to polymeric speecies present at the higher concentration of 50 μM that was necessary for routine NMR spectroscopy. In addition, because of the much narrower concentration range of KOH required to shift the spectrum from that characteristic of the monoanion

Table I: Identification Criteria for Some of the Vanadates Studied[a]

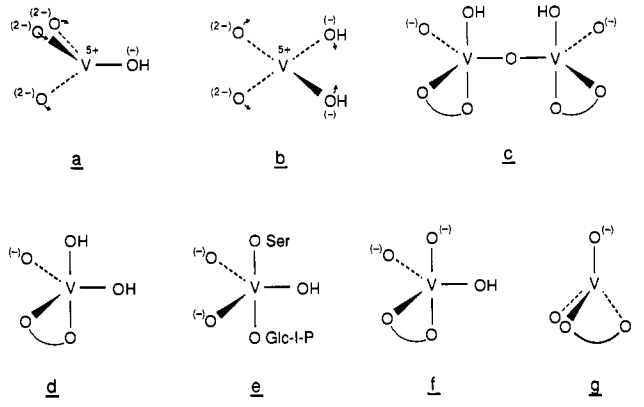
species	medium[b]	conditions of examination/criteria for identification[c]
$(HO)_2VO_2^-$	20 mM Im, pH 7	reported pK_a values (Kepert, 1973); concn ind molar abs (10–50 μM); SR-NMR (−561 ppm)
	97% DMSO, 0.1 M 18-C-6	spec titration (Im/Im·HCl = 1/1 ↔ CAPS/KOH = 1/0.9); concn ind molar abs (10–20 μM); SR-NMR (−550 ppm)
	94% dioxane, 15 mM CHES, 0.1 M 18-C-6	spec titration (2.5 mM KOH/37.5 mM KCl ↔ 12.5 mM KOH/27.5 mM KCl); small, concn dependency of apparent molar abs (5–10 μM); NMR major resonance, −517.5 ppm (broad, upfield peaks develop with time)
	8.6 M GCl, 20 mM MES, 20 mM Im	spec titration, pH 6–8.6 (pK_a^{app} = 7.0)
$HOVO_3^{2-}$	20 mM CAPS, pH 10.8–11.0	reported pK_a values (Kepert, 1973); concn ind molar abs (10–50 μM); SR-NMR (−534 ppm)
	97% DMSO, 0.01 M 18-C-6	spec titration, 0.4–3.8 mM KOH (1 mM KOH ↔ 2 mM KOH); concn ind molar abs (10–50 μM);[d] SR-NMR (−524 ppm)[e]
	94% dioxane, 0.08 M 18-C-6	spec titration, 1–20 mM KOH (8 mM KOH ↔ 8 mM KOH);[f] SR-NMR (−541 ppm)
	8.6 M GCl, 20 mM MES, 20 mM Im	spec titration, pH 6–8.6 (pK_a^{app} = 7.0); observed at pH 8.6
	4 M $CaCl_2$, 20 mM MES, 20 mM Im	spec titration, pH 5.5–8.8; observed at pH 8.8
VO_4^{3-}	2 N NaOH	reported pK_a values (Kepert, 1973)
$(CH_3O)_2VO_2^-$	80–99% methanol di-Me_4G salt	lack of dependence on methanol concn of spectra obtained with unbuffered solutions; identity with spectrum in 80% methanol, 10 mM imidazole/10 mM imidazolium chloride
$(tBuO)_2VO_2^-$	99% *t*-Bu, 0.1 N di-Me_4G	SR-NMR −609 ppm (−597 ppm in water; Tracey & Gresser, 1988)
$CH_3OVO_3^{2-}$	99% methanol, 0.32 M KOH	spec titration, 0.32–1 M KOH (0.1 M KOH ↔ 0.32 M KOH); SR-NMR −530 ppm (−528 ppm in water; Tracey & Gresser, 1988)

[a] Esters in largely aqueous solutions were identified in terms of the composition of the medium and published formation constants (see Experimental Procedures). [b] Abbreviations: Im, imidazole; 18-C-6, 18-crown-6 ether; GCl, guanidinium chloride; Me_4G, tetramethylguanidine. [c] Some identifications of ionic species are based on spectral titrations, usually at 10 μM total vanadate, through a "ledge" region bounded by decreased absorbance at lower base strength and increased absorbance at higher base strength. In such cases the approximate width of the ledge is identified by (low ↔ high). Spectra whose intensities increase by n(1 ● 0.03)-fold with an n-fold increase in vanadate concentration are considered concentration independent and are designated by "concn ind molar abs". SR-NMR designates a single ^{51}V NMR resonance in the range −445 to −635 ppm from $VOCl_3$ obtained at a V_i concentration of 50 μM. [d] Because of the narrowness of the observed ledge (see *c*), both monoanionic species and the trianion make contributions to the observed spectrum. However, traces of acidic material present in the best grade of DMSO available also contributed to the narrowness of the ledge. [e] An upfield resonance at −596 ppm also was observed. Even with the best available grade of DMSO, this resonance increased with time over a period of several hours as the solution yellowed, presumably as the result of a slow redox reaction. [f] No real ledge observed, only an inflection.

to that characteristic of the trianion than is required in aqueous solution, spectra of the dianion in both dioxane and DMSO may include contributions from both the *mono-* and the *tri-*anion (see footnote 4). In spite of these caveats, neither the NMR results nor the ultraviolet spectra in Figure 1b provide any indication that water is an intrinsic part of the structure of the vanadate *mono-* or *di*anion. Additional spectral comparisons of esters in largely aqueous solution and in essentially anhydrous alcohols (see below) support this conclusion. As a corollary, a simple replacement of the solvating water around a vanadate ester by less efficient ligands will not produce a V(V) spectrum similar to that observed for the transition-state-analogue complexes of phosphoglucomutase.

Spectra of Inorganic Vanadate(V) in Solutions of High Ionic Strength. Figure 1c shows that no large changes in the spectrum of $HOVO_3^{2-}$ and $(HO)_2VO_2^-$ occur in 8.4 M guanidinium chloride (mole ratio salt to water, 1:2.3) or in 4 M $CaCl_2$. $MgCl_2$ at a concentration of 4 M does not produce a substantial effect, either, although a high enough pH to convert all of the vanadate present to the dianion could not be obtained because of precipitation of $Mg(OH)_2$. However, at pH 6.4, where approximately 80% of the vanadate was present as the dianion in the case of $CaCl_2$, the spectrum of vanadate was essentially the same in 4 M $CaCl_2$ and 4 M $MgCl_2$. Thus, any distortion of the ligand sphere of V(V) from tetrahedral symmetry in $HOVO_3^{2-}$ and $(HO)_2VO_2^-$ that *might* arise because of an asymmetrical distribution of electrostatic charge (cf. parts a and b, Chart I) cannot be substantially affected by external charge shielding at high ionic strength or by metal ion binding. As a corollary, the binding of a vanadate ester at a region of high effective ionic strength at the active site of an enzyme is unlikely to produce the type of spectral changes observed in Figure 1a—at least when ion–ion interactions between the bound vanadate and cations

Chart I: Structural Representations of Various Vanadate Diesters[a]



[a] Here, the dianion of a 1,2-glycol is represented as $\overline{O^- \quad O^-}$. (a) and (b) are schematics showing the possible geometrical effect of the asymmetric charge distribution in the ligand sphere of four-coordinate vanadate di- and monoanions, respectively. (c) is a dimer of a 1,2-glycol cyclic diester monoanion. (d) is a monomeric form of (c). (e) is a pentacoordinate adduct that might be present in the transition-state-analogue complexes of phosphoglucomutase. (f) is a hydroxide adduct of (g). (g) is a tetrahedral cyclic diester of a 1,2-glycol whose water adduct is (d) and whose hydroxide adduct is (f).

at the active site of the enzyme mimic those in solution. In fact, both the association of Ca^{2+} and guanidinium ion with $HOVO_3^{2-}$ produce effects opposite to those observed on binding of V-6-Glc-1-P to phosphoglucomutase, viz., modest blue shifts with a small decrease in molar absorbance.

Effect of Ester Formation on Spectra of the Monoanion and Dianion of Vanadate(V). The spectra in parts d–f of Figure 1 were obtained to determine whether either *internal* steric effects or electronic effects in vanadate esters might produce significant changes in the spectra of the *mono-* and

*di*anions, respectively. However, it should be stated in advance that these spectra are intended only to identify general trends. Thus, the molar spectra in parts d and f of Figure 1 were obtained by resolution of the spectra of multicomponent systems (usually three components in the case of the *mono*anion and two in the case of the *di*anion). In addition, published equilibrium constants that are given only to one significant figure were used—frequently at alcohol concentrations substantially different from that for which the equilibrium constants are reported (see Experimental Procedures). Thus, in the solution of the simultaneous equations required to define the concentration of a single species in a mixture, the error of the result depends critically on one or two spectral differences (numerator terms) and a denominator factor, both of which are functions of alcohol concentration, molar extinctions, and equilibrium constants. Concentrations of alcohol always were employed so that the denominator term was at least 0.15 (and greater than 0.25 where possible), and the spectral difference in the numerator was at least 0.15 of the least intense spectrum.

Such an approach assumes that the spectra of both V_i and the esters in question are independent of alcohol concentration. In view of the relatively minor spectral changes produced by 98% dimethyl sulfoxide and 93–94% dioxane (Figure 1b), the assumed lack of a solvent-induced spectral effect for both V_i and vanadate esters seems reasonable, since in no case was a spectral resolution attempted for a solution in which the alcohol concentration exceeded 50% (usually 40% was the limit). In addition, the "single-component" spectrum of $CH_3OVO_3^{2-}$ obtained in 99% methanol (Figure 1f) and that calculated from the spectrum of a two-component mixture at 33% methanol (not shown) were virtually identical. Spectra of $(CH_3O)VO_2^-$ and $(C_2H_5O)_2VO_2^-$ obtained in anhydrous alcohol (Figure 1e) and those calculated from spectra of largely aqueous solutions of alcohol (not shown) were similar, even though the diesters were minor components of the mixture and the denominator term noted above was too small to allow precise results to be obtained in the aqueous solution.

By contrast with the procedure used for obtaining the spectra in parts d and f of Figure 1, spectra of monoanion diesters were obtained in essentially anhydrous alcohol in the presence of 20–40 mM tetramethylguanidine. In the case of methanol, addition of up to 20% by volume of water produced no significant spectral changes, while in the case of *tert*-butyl alcohol, a small but definite change was observed on addition of as little as 2% water. Intermediate results were observed for ethanol and 2-propanol. Some of the criteria used to conclude that the diester of vanadate was the species present under the conditions used are given in Table I.

In spite of the caveats noted above for spectra of the monoester monoanions in Figure 1d and of monoester dianions in Figure 1f, the trends in the results are clear: increasing intensity with a modest accompanying red shift as HO^- in the coordination sphere of V(V) is replaced by larger and more basic alkoxides, e.g., CH_3O^-, $C_2H_5O^-$, $(CH_3)_2CHO^-$, and $(CH_3)_3CO^-$.[6] (Note that the absorbance scale for spectral plots of vanadate esters in parts d–f of Figure 1 is 1.6-fold larger than for other plots in Figure 1.) While much larger intensity effects are produced in the series of *mono*anions than in the *di*anions, most of the increased intensity seems to arise from a red shift in absorbance bands at wavelengths below those accessible with most of the solvent systems used.[5] To what extent the trends observed as the identity of the R group is altered in the above series are steric in origin or arise exclusively from electronic effects cannot be determined, although spectra for esters of the relatively acidic alcohols, CF_3CH_2OH and $(CF_3)_2CHOH$, appear to follow the general trend of pK_a-related effects established by the much more basic nonfluorinated alcohols.[7] [$(CF_3)_2CHOH$ is too acidic to allow formation of the ester dianion.] In any case, neither steric effects of the type provided by the *t*-Bu group in the monoanionic systems nor the unlikely formation of a diester with a hydroxyl group of the enzyme can be invoked to provide a rationale for the spectra of the vanadate-based transition-state-analogue complexes of phosphoglucomutase. (Identification of the vanadate species present in basic solutions of 80–93% *tert*-butyl alcohol, where the monoester dianion should have represented a substantial fraction of the vanadate present, proved excessively difficult.[8])

Spectra of Pentacoordinate Complexes of Vanadate(V). Figure 1g shows the spectra of four pentacoordinate complexes of vanadate that are cyclic diesters of vicinal diols: 2,3-butanediol, *cis*- and *trans*-cyclohexanediols, and β-methyl riboside. While the equilibrium constants for the formation of such complexes are large enough that essentially a single species is present at the concentrations used [see figure legend and constants reported in Gresser and Tracey (1986) and Tracey et al. (1988a)], direct comparisons with the spectra of these pentacoordinate complexes is complicated by the fact that all three are dimeric (Gresser & Tracey, 1986; Tracey & Gresser, 1988b), viz., they contain a central V–O–V bond (see part c, Chart I). The oxygen in the V–O–V linkage (see below) has different electronic properties from those of the oxygen ligands in other models studied here that should partially offset the blue shift expected for conversion of a tetrahedral complex of V(V) to a pentacoordinate complex. Hence, the *monomeric* forms of these complexes are expected to absorb at shorter wavelengths than observed for the corresponding *dimers*, which in general already exhibit absorbance peaks at somewhat

[5] Cr(VI) exhibits two electron-transfer absorbance bands, at about 370 and 260 nm (Schatz et al., 1966). The longer wavelength band likely corresponds with that of V(V) at about 270 nm. Hence, a shorter wavelength electron-transfer band for V(V) is expected. This band should respond to the substitution of RO^- for HO^- in a manner similar to that of the 270-nm band, viz., should exhibit an increased red shift and an increased intensity with increased substitution of more basic RO^- groups. It is likely that a red shift in the longer wavelength components of this band produces the enhanced absorbance observed in spectra of the monoanion at wavelengths below 250 nm.

[6] The terms ligand and coordination sphere are used in the following context. Although compounds of the type $ROVO_3^{2-}$ can be considered esters, since vanadium is a metal ion, they also can be treated as coordination complexes: $V^{5+}(RO^-)(O^{2-})_3$. Since frequent reference is made herein to crystal field theory, which deals with geometrically disposed charge–charge interaction, we refer to groups attached to vanadium as "ligands of V(V)", while retaining the former structural representation, because of its analogy with phosphate chemistry.

[7] The possibility that geometrical changes are produced in the ligand sphere of vanadate *mono*- or *di*anions by substitution of bulky RO^- ligands for the HO^- ligand is unattractive in view of the observation that successive substitution of CH_3 groups for methyl hydrogens of $CH_3OVO_3H^-$, $(CH_3O)_2VO_2^-$, or $CH_3OVO_3^{2-}$ produces incremental changes in the respective ^{51}V NMR chemical shifts that are similar in magnitude [except in the case of $(t\text{-}BuO)_2VO_2^-$, where the chemical shift was measured in a different solvent system (Tracey & Gresser, 1988a)].

[8] A substantial fraction of V_i in 81% *tert*-butyl alcohol and 5 mM KOH (about 20% of the total) is expected to be present as the dianion ester on the basis of equilibrium constants reported for a 10% solution (Tracey et al., 1988a). However, only one ^{51}V NMR resonance was observed under these conditions, and that resonance was substantially closer to the chemical shift of $HOVO_3^{2-}$ than that reported for *tert*-$BuOVO_3^{2-}$ (Tracey & Gresser, 1988a). Even at 94% *tert*-butyl alcohol (4 mM KOH/50 mM 18-crown-6 ether), only a single NMR resonance was observed. We have no rationale for these observations.

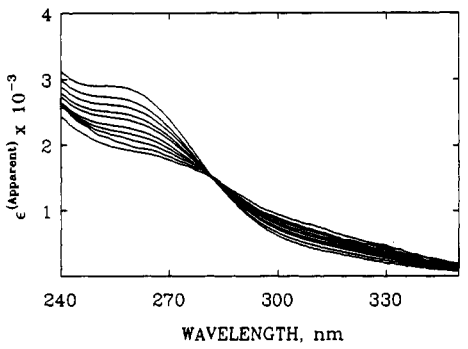


FIGURE 2: Normalized spectra of vanadate in the presence of β-methyl glucoside at different vanadate concentrations. Spectra were obtained at 0.78–240 μM vanadate in the presence of 0.12 M β-methyl glucoside and 20 mM imidazole/imidazolium chloride, pH 7. Results are presented in terms of the weighted average molar absorbance of all vanadates present. From top to bottom (at the left) the spectra were obtained as follows: by extrapolation to infinite vanadate concentration, at 120, 30, 15, 7.5, 3.7, 1.78, 0.97, and 0.47 μM vanadate, and by extrapolation to zero vanadate concentration, respectively.

shorter wavelengths than the acyclic diester involving methanol (see Figure 3e).

One pentacoordinate complex of vanadate that is monomeric is the transition-state-analogue complex which ribonuclease forms when it is treated with uridine and vanadate (Lindquist et al., 1973; Wlodawer et al., 1983; Borah et al., 1984). Unfortunately, only a portion of the spectrum due to the oxyvanadium chromophore in this complex could be collected when the concentration of uridine was high enough (10 μM) to ensure that essentially all of the added vanadate is present as the RNase/uridine/vanadate complex [W. J. Ray, Jr.; calculation based on constants reported by Lindquist et al. (1973) and by Tracey et al. (1988c)]. This partial spectrum also is given in Figure 1g. Except for increased intensity in the long-wavelength portion of the spectrum, which likely is caused by the decreased degeneracy of V(V) orbitals in a distorted pentacoordinate complex (see Discussion), there is no indication that the missing part of the spectrum would deviate markedly from the spectra of the dimeric pentacoordinate vanadate derivatives also shown in the same figure.

Another monomeric pentacoordinate complex of vanadate is that formed by the reaction of the monoanion with 2 equiv of lactate. The spectrum of this complex obtained under conditions where the pentacoordinate species was present almost exclusively [2 M lactate, pH 7.3; see Tracey et al. (1987)] resembled that of the RNase/U/V_i complex more closely than the dimeric complex described above, due to the increased prominence of the long-wavelength portion of the spectrum. This spectrum is not shown because the carboxylate group is rather different from the oxy ligands of other models studied here.

Binding of Vanadate(V) to β-Methyl Glucoside: Formation of a Pentacoordinate Complex. Figure 2 shows apparent molar extinction spectra of the vanadate species present in a solution of 0.12 M β-methyl glucoside and 20 mM imidazole, pH 7, as a function of total vanadate concentration [V_T], from 0.78 to 250 μM. The isosbestic point at about 280 nm, plus the plot described below, is consistent with an equilibrium of the type

$$2(V_1 \rightleftharpoons V_2 \rightleftharpoons ::: \rightleftharpoons V_n)_M \xrightleftharpoons{K_{M,D}} V_D \qquad (1)$$

where $(V_1 + V_2 + ... + V_n)_M$ are monomeric forms of vanadate (see Scheme I) and V_D is the dimer of the (pentacoordinate) cyclic diester of vanadate (species c, Chart I). Thus, a semilog plot of the weighted average molar extinction of all V(V) species present, $\epsilon^{apparent}$ (which should be equal to $\epsilon(V_D)/2 + \sum\epsilon(V_n)_M$), versus the total vanadium concentration, V_T (× and +, Figure 3), conforms closely with that expected for eq 2 when $K^{app}_{M,D}$ is taken as 1.3×10^5 M (solid line) over a range of V_T concentrations that exceeds 300-fold.

$$K^{app}_{M,D} = [V_D]/([V_T] - 2[V_D])^2 \qquad (2)$$

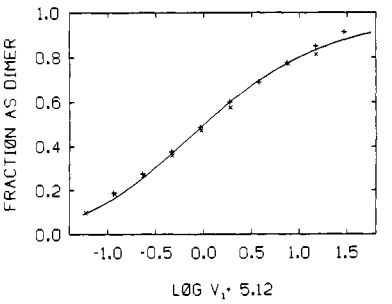
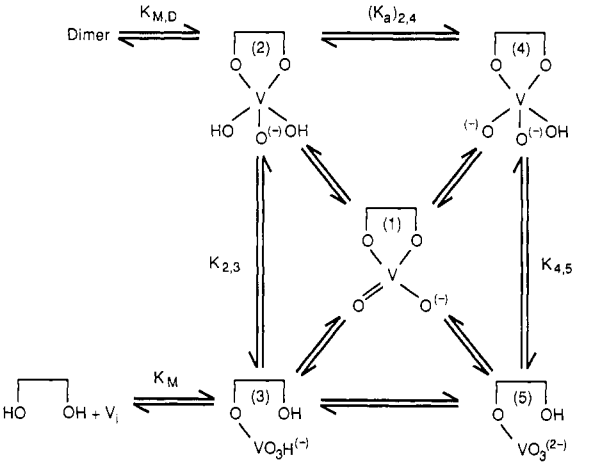


FIGURE 3: Fractional change in weighted average molar extinction of all vanadate present under the conditions described in Figure 2. Values of weighted average molar extinction from Figure 2 were fit to a variation of eq 2 with $K^{app}_{M,D} = 1.3 \times 10^5$ M: (×) data obtained at 255 nm; (+) data obtained at 310 nm.

Scheme I: Monomeric Forms of Vanadate in Equilibrium with the Pentacoordinate Cyclic Dimer That Vanadate Forms with Glycols (Cf. Chart I)



Since the structural identity of the β-methyl riboside/vanadate dimer has been established by the ^{51}V NMR studies of Tracey and Gresser (1988b) and Tracey et al. (1988c), the monomeric species that actually dimerizes must be the pentacoordinate cyclic diester (2, Scheme I). Unfortunately, sufficient data to rigorously evaluate $K_{M,D}$ are not yet available, since such an evaluation would require a knowledge of the equilibrium constants for all of the equilibria in Scheme I. However, on the basis of the equilibrium constant for $2V_i + 2U \rightleftharpoons U_2V_2$, 2.8×10^7 M^{-3} (Tracey et al., 1988c), where U is uridine and U_2V_2 is the dimer analogous to structure c of Chart I, plus other related constants that also are published, and the assumption that $K_{M,D}$ is essentially the same in the uridine and β-methyl glucoside systems, we estimate a value of 10^6 M^{-1} for $K_{M,D}$, which would thus apply to both systems (calculations available on request from W.J.R.).[9] The limiting

[9] We also estimate that the value of $K_{2,3}$ (Scheme I) is about $^1/_{15}$ for the 2- and 3-hydroxyl groups in the ribose system (calculations available on request from W.J.R.). With this value, a value of 0.4 M for K_M in Scheme I (estimated from published equilibrium constants for related processes; Tracey & Gresser, 1988b), the value of 3.9×10^5 M for $K^{app}_{M,D}$, noted above, and the inhibition data of Lindquist et al. (1973), K_I was estimated as 2 μM if the form of the complex bound to ribonuclease is the pentacoordinate cyclic diester monoanion (species 2, Scheme I). If it is the corresponding dianion (species 4, Scheme I), K_I would be smaller—perhaps as small as 20 nM.

spectrum at high vanadate, viz. that of the riboside/vanadate dimer, is shown in Figure 1g along with the spectra of other pentacoordinate vanadate dimers.

An Octahedral Complex of Vanadate(V). The vanadate monoanion forms an octahedral complex with two pyrosphosphate molecules. The spectrum of this complex, obtained under conditions where it should be essentially the only form of vanadate present [20 or 100 mM pyrophosphate, pH 7; μ = 1.0: see Gresser et al. (1986)], resembled that of the monoanion, but with a long-wavelength shoulder of increased prominence and a shift to shorter wavelengths for both absorbance bands (see Figure 1h). The bisoxalate complex of vanadate also is octahedral (Tracey et al., 1987); however, the oxalate ligand absorbs in the near ultraviolet, and the spectrum of this complex is not described further.

Complexes with Compounds Containing Thiol Groups. A solution containing 1 mM V_i and 0.1 M mercaptoethanol or 0.1 M cysteine in imidazole buffer, pH 7.5, was diluted 40-fold in the same buffer and the ultraviolet spectrum compared with that of a 25 μM solution of V_i that contained 2.5 mM mercaptoethanol or 2.5 mM cysteine. The spectrum of the V_i in the latter two solutions, where no complex is expected, was that characteristic of V_i alone, while the spectrum of V_i previously exposed to the higher concentrations of the above thiols was entirely different and remained unchanged on long standing at the more dilute concentration. Apparently V_i is reduced by such compounds since the NMR signal of V_i also rapidly "disappears" under similar conditions (M. Gresser, personal communication).

Comparison of Spectra of the Oxyvanadium Constellation in the Mg(II) and Li(I) Complexes of Phosphoglucomutase (Dephospho Form), Glucose 1-Phosphate, and Vanadate. Figure 1i (lower spectrum) shows the spectrum of the oxyvanadium constellation in the E·V-6-Glc-1-P(Li^+) complex [from the second paper in this series (Ray et al., 1990)]. The analogous spectrum for E*V*6-Glc-1-P(Mg^{2+}) is reproduced from Figure 1a for comparison. The overall intensity of the V(V) spectrum in the Li^+ complex is consistent with that of a dianionic vanadate, which is in accord with the ionic status of the corresponding phosphate in E·P-6-Glc-1-P(Li^+) (Rhyu et al., 1985). Since water does not interact directly with either phosphate group in the latter complex (Rhyu et al., 1984), we assume the same holds for the vanadate analogue complex. In addition, studies described in the fourth paper of this series suggest that the oxyvanadium constellation is much less distorted in the complex involving Li^+, as opposed to Mg^{2+}, which is the basis of our use of · instead of * in representing the Li^+ complex. Hence, the broad spectrum in Figure 1i likely is that of a modestly distorted, tetrahedral vanadate dianion in a nonaqueous environment.

Discussion

The second paper in this series (Ray et al., 1990) shows that in the vanadate-based "transition-state-analogue" complexes of phosphoglucomutase the vanadate group of the mixed phosphate/vanadate 1,6-diesters of glucose is bound at the proximal site, where (PO_3^-) transfer normally occurs. Hence, it is appropriate to inquire whether the greatly increased stability of these complexes, E*V*6-Glc-1-P and E*V*1-Glc-6-P, relative to E·P-6-Glc-1-P and E·P-1-Glc-6-P [see the fourth paper in this series (Ray & Puvathingal, 1990)] actually is due to the formation of an adduct between the bound vanadate ester group and the hydroxyl group of the active-site serine (Ser^{116}; Ray et al., 1983), as is suggested by Percival et al. (1990). This inquiry is based on the unusual ultraviolet spectrum of V(V) in the oxyvanadium constellation of these complexes (Figure 1a) and considers whether tetrahedral vanadate in an unusual environment or a pentacoordinate vanadate complex might exhibit such an absorbance spectrum. Since both phosphate groups of bound glucose bisphosphate appear to be dianionic (Rhyu et al., 1985), V-6-Glc-1-P and V-1-Glc-6-P presumably bind as tetraanions also. Hence, we are concerned primarily with dianionic vanadates. However, a number of monoanionic vanadates also were studied for comparison.

The near ultraviolet spectrum of vanadate(V) derivatives is produced by electron transfer from an orbital primarily associated with a ligand in the coordination sphere of the central V(V) ion[6] to a vacant orbital primarily associated with the 3d shell of the metal. [In the ground state the 3d shell of V(V) is vacant.] In general, electron transfer of this type is facilitated when more polarizable ligands or ligands with increased electron-donating capacity make up the ligand sphere of the metal ion and red shifts accompany such substitutions. Alternatively, decreasing the coordination number of the central metal ion, as in the series MX_6, MX_4, MX_2, also facilitates electron transfer by lowering the energy of the d orbitals of the metal ion (Lever, 1974).

However, in the case of V(V) uncertainties about the precise structure of simple vanadates cloud the picture. Thus, the basis for the marked decrease in extinction coefficient of VO_4^{3-} that is produced by protonation to give $HOVO_3^{2-}$ and $(HO)_2VO_2^-$ (Figure 1a) not only is unknown but is unexpected in terms of simple models. Since VO_4^{3-} is the only one of these ions that is known to be tetrahedral in solution (Griffith & Wickins, 1966), the observed proton-induced decrease in extinction and modest blue shift might be caused by an increased hydration by water. Thus, the dianion might be $(HO)_3VO_2^{2-}$ [cf. Cotton and Wilkinson (1967)] and the monoanion might be $(H_2O)(HO)_4VO^-$, as has been posed by Tanaka and co-workers (Yamada et al., 1975; Yuchi et al., 1979), although others have argued against such hydrated structures (Pope & Dale, 1968; Kepert, 1973). In fact, analogous spectral changes accompany similar reactions of the neighboring metal ion, Cr(VI): $CrO_4^{2-} + H^+ \rightarrow HOCrO_3^-$, and $CrO_4^{2-} + H^+ + HCl \rightarrow ClCrO_3^- + H_2O$. Spectral changes accompanying the latter process have been rationalized in terms of the more complex ligand field theory without recourse to a change in coordination (Aymonino et al., 1969). Still, because vanadates exhibit an propensity toward forming five- and six-coordinate complexes, the possibility remains that a change in coordination involving water does accompany protonation. If so, the spectrum of V(V) in the inhibitor complexes might be rationalized by a decrease in coordination due to reversal of hydration on binding to the enzyme. However, on the basis of results obtained in largely nonaqeous solvents (e.g., 98% dimethyl sulfoxide, 94% dioxane, or anhydrous alcohols), it appears that water is not an integral part of the coordination sphere of either the *mono-* or the *di*anion of vanadate(V). Hence, the removal of the vanadate ester grouping from a completely aqueous environment upon binding to phosphoglucomutase[10] (plus whatever minor geometrical reorganization of the ligand sphere might accompany such changes in solvation; cf. structures a and b, Chart I), is far from sufficient to provide a rationale for the spectrum of V(V) in the transition-state-analogue complexes.

Crystal field theory indicates that increasing the polarizability of ligands should produce a red-shifted absorbance spectrum for V(V), and such a shift is observed for the V-6-Glc-1-P complexes of phosphoglucomutase. Hence, a number

[10] ^{31}P NOE studies (Rhyu et al., 1984) suggest that water does not solvate the phosphate groups in the E·Glc-P_2(Li^+) complex.

of different esters of both the *mono-* and *di*anion of vanadate were examined to obtain an idea of the magnitude of the shifts that might be obtained in this way. Both *mono-* and *di*esters of the *mono*anion, $(RO)(HO)VO_2^-$ and $(RO)_2VO_2^-$, respectively, absorb at longer wavelengths than the parent monoanion. In addition, when ROH is more basic than HOH, the absorbance is more intense, while more acidic alcohols produce less intensely absorbing esters (Figure 1d,e). A similar trend is observed for esters of the dianion (Figure 1f), although the absorbance of the ester with CF_3CH_2OH is not reduced below that of $HOVO_3^{2-}$. But in all cases both shifts in wavelength and intensity changes are much smaller than those observed on formation of the transition-state-analogue complexes. In addition, neither transfer of $(HO)_2VO_2^-$ or $HOVO_3^{2-}$ to media of very high ionic strength nor binding of bivalent metal ions by the dianion produces substantial spectral changes (Figure 1c). In fact, what changes are observed are in the direction of reduced intensity and shorter wavelengths and thus are in the wrong direction to provide a rationale for the spectra of the enzymic complexes. While transfer of $HOVO_3^-$ and $(HO)_2VO_2^-$ to less polar solvents does produce a spectral red shift, the size of the effect, even in 94% dioxane, is relatively small. Hence, we conclude that in solution it is unlikely that a simple, tetrahedral vanadate will exhibit a spectrum analogous to that of the transition-state-analogue complexes of phosphoglucomutase and, as a corollary, that the oxyvanadium constellation in these complexes is not that of a regular tetrahedral complex of V(V). Thus, consideration of pentacoordinate complexes of vanadate is of particular importance.

According to crystal field theory a shift to shorter wavelengths should accompany the process $ML_4 \rightarrow ML_5$ when the same type of ligand is involved. However, not only does V(V) in V-6-Glc-1-P contain a mixture of ligand types, but formation of a pentcoordinate complex between the inhibitor and the active-site serine residue of the enzyme would change this mixture, as in $(RO)VO_3^{2-} + R'OH \rightarrow (RO)(R'O)(HO)VO_2^{2-}$ (see structure e of Chart I). Because systems with mixed ligands are difficult to treat theoretically, a suitable model for pentacoordinate V(V) is particularly desirable. However, in most cases only the dimeric forms of pentacoordinate diester complexes of V(V) are known in aqueous solution. [Usually these are cyclic diesters of vicinol diols where cyclization and dimerization are tightly coupled (M. Gresser, personal communication; W.J.R., unpublished results).] Moreover, no compound analogous to the dianionic adduct is known, even in dimeric form. In fact, there is a good reason to believe that compounds of this type would be unstable, since $(RO)_2(HO)VO_2^{2-}$ (structure e of Chart I), as well as the dimer thereof, can be considered the hydroxide adduct of a vanadate diester monoanion (structures f and g, respectively, of Chart I) and almost certainly would exhibit a marked tendency toward hydrolysis to the monoester.[11]

On the other hand, the formation of cyclic pentacoordinate adducts involving monoanion monoesters of glycols is well established (Gresser & Tracey, 1986; Tracey & Gresser, 1988b; Tracey et al., 1988c). The ultraviolet spectra of four such complexes were examined, in spite of the fact that all are monoanions and that all are dimers with a central V–O–V bond. The near ultraviolet absorbance maximum of these pentacoordinate dimers occurs at wavelengths similar to or shorter than that of $(RO)_2VO_2^-$, although the further decrease in d orbital degeneracy (relative to $ROVO_3^{2-}$) that accompanies formation of the pentacoordinate adduct, plus the effect that decreased degeneracy has on spectral tailing at the longer wavelengths, precludes definite assignments of peak position. In any case, the corresponding pentacoordinate *monomers* should absorb at shorter wavelengths than the *dimers*, and the process for formation of a pentacoordinate complex should be accompanied by either a spectral blue shift or no significant shift.

In a corresponding manner, formation of the analogous pentacoordinate cyclic diester dianion in the dianionic system, $(RO)VO_3^{2-} + R'OH \rightarrow (RO)(R'O)(HO)VO_2^{2-}$, also should be accompanied by a spectral blue shift or no significant shift. Since V(V) in the transition-state-analogue complexes absorbs at a considerably *longer* wavelength than $ROVO_3^{2-}$, we conclude that the vanadate grouping in the analogue complexes is not pentacoordinate (see below). [The probable requirement that both RO^- ligands of any pentacoordinate adduct that might form at the active site of the enzyme be apical, instead of apical/equatorial (see structures d and e of Chart I) as in the cyclic pentacoordinate diesters of Figure 1g, is not expected to have a substantial effect on absorbance properties of V(V).]

Since our attempts to mimic the spectrum of V(V) in the transition-state-analogue complexes of phosphoglucomutase involved a relatively broad examination of reasonable models, and since these attempts were unsuccessful, using crystal field theory to provide a rationale for the spectrum in question seems in order. Thus, the electron-transfer spectra for a number of transition-metal ions with no d electrons is reasonably well understood (Jorgenson, 1970; Lever, 1974), and a number of our spectral observations of V(V) can be explained in terms of this theory, for example, the longer wavelength shoulder when the trianion is converted to the dianion or the dianion ester is produced by the decreased degeneracy of the d orbitals of V(V). In addition, the red shift when HO^- is successively replaced by RO^- of increasing basicity in both $(HO)_2VO_2^-$ and $HOVO_3^{2-}$ and the modest blue shift on coordination of the peripheral oxygens by a metal ion are expected results of polarizability changes. While the much more complex but more realistic ligand field theory would be required for any serious attempt to quantitate spectral changes such as those observed on protonation of VO_4^{3-}, the simpler theory should suffice to provide clues as to how the large spectral red shift for V(V) in the transition-state-analogue complexes, relative to $ROVO_3^{2-}$, might arise.

As is noted above, a basic tenet of crystal field theory is that the "pressure" on the d orbitals of the central metal ion from the electrons of ligands in the coordination sphere of the metal ion increases the energies of electrons that are transferred to those orbitals by absorption of light, as in the generation of the ultraviolet spectra described here. Hence, we argue that *reduced* V(V)–ligand interactions, relative to $ROVO_3^{2-}$, produce the spectral red shift observed for the enzymic complexes. The enzyme might reduce ligand–metal ion interactions in three ways: by protonating one or more oxygen ligands, by forming H bonds with the oxygens of $ROVO_3^{2-}$ that are stronger than those in aqueous solution, or by causing the vanadate group to bind to a metal ion (e.g., Mg^{2+}). However, as is noted above, in reasonable models none of these processes produce spectral shifts that are comparable in magnitude to that observed for the transition-state-analogue complexes of phosphoglucomutase. Hence, by default, we suggest that the red shift in the spectra of the enzymic complexes is produced

[11] In spite of theory, an attempt was made to obtain evidence for such an adduct by monitoring absorbance changes as the pH of a solution containing the dimer of the pentacoordinate cyclic diester of $(HO)_2VO_2^-$ and butane-2,3-diol was increased from 7 to 12. However, the results indicate the presence of only two species, the starting dimeric diester and the monomeric monoester dianion.

by a significant weakening of the V–OR bond. Such bond weakening in the E*V*6-Glc-1-P complex, which would produce an effective coordination for vanadium of less than four, is attractive in spite of other results that are difficult to reconcile with such a process [Ray et al., 1990; see also the fourth paper in this series (Ray & Puvathingal, 1990)]. The attraction arises because the transition state for model (PO_3^-)-transfer processes involving dianionic phosphates also involves a reduced coordination—here with respect to the central phosphorus—because of the substantial breaking of the P–OR bond with only minimal bond formation relative to the entering group (Allen & Haake, 1980; Bourne & Williams, 1984; Skoog & Jencks, 1984; Jencks, 1985; Herschlag & Jencks, 1987, 1988a,b; Williams, 1987).

The difference between the above model for the transition-state-analogue complexes of phosphoglucomutase and that suggested by Percival et al. (1990) should be emphasized. In Percival's model, V(V) is surrounded by five ligands, and the analogue complexes are stabilized by *increased* bonding of oxygens to V(V)—as in the formation of a cyclic pentacoordinate diester adduct of vanadate with the 2- and 3-hydroxyl groups of β-methyl glucoside (structure e of Chart I). By contrast, we suggest that although V(V) in the analogue complexes probably does have five close neighbors, a net *decrease* in bonding between V(V) and the surrounding oxygens is produced on binding. How this might occur is considered in the fourth paper in this series (Ray & Puvathingal, 1990).

A number of related observations can be made about the ribonuclease/uridine/vanadate system, where the formation of a pentacoordinate complex has been verified by X-ray and neutron diffraction (Wlodawer et al., 1983; Borah et al., 1984). In the study of Lindquist et al. (1973), where the formation of an inhibitor complex involving the above three species was first demonstrated, the binding constant for the uridine/vanadate adduct was calculated on the basis of an erroneous solution stoichiometry [cf. Tracey et al. (1988c)]. In fact, the predominant vanadate-containing species (other than V_i) in the solution used for those inhibition studies was the dimeric form of the pentacoordinate uridine/vanadate adduct (calculations based on studies described herein), and it is not the dimer that is bound to RNase under these conditions (M. Gresser, personal communication; A. Wlodawer, personal communication). This means that although the general structure of the vanadate-based transition-state-analogue complex of ribonuclease is relatively well established, the state of protonation and thus the concentration of the uridine/vanadate species that is in equilibrium with the bound inhibitor are not. Hence, the affinity of RNase for the species that actually binds to produce the transition-state-analogue complex also is not known but is substantially higher than was estimated originally.

In any case, the difference in the way ribonuclease and phosphoglucomutase appear to stabilize vanadate-based models of their respective transition states is interesting. While ribonuclease selects a pentacoordinate species and thereby increases the coordination number of vanadium, phosphoglucomutase appears to reduce the effective coordination to less than four. Indeed, this difference may turn out to be characteristic of enzymic transfer reactions involving phosphodiesters as opposed to phosphate ester dianions, although the extent to which binding interactions characteristic of the transitions state for the reaction of either enzyme are reflected in the complexes studied cannot be estimated as yet. However, the interactions that phosphoglucomutase utilizes to bind V-6-Glc-1-P are so strong that it would be surprising if phosphoglucomutase did not use analogous interactions to stabilize the transition state for its reaction [cf. the fourth paper in this series (Ray & Puvathingal, 1990)].

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