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Reduction of $[VO_2(ma)_2]^-$ and $[VO_2(ema)_2]^-$ by Ascorbic Acid and Glutathione: Kinetic Studies of Pro-Drugs for the Enhancement of Insulin Action†

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To shed light on the role of V(V) complexes as pro-drugs for their V(IV) analogues, the kinetics of the reduction reactions of $[VO_2(ma)_2]^-$ or $[VO_2(ema)_2]^-$ (Hma = maltol, Hema = ethylmaltol), with ascorbic acid or glutathione, have been studied in aqueous solution by spectrophotometric and magnetic resonance methods. EPR and ^{51}V NMR studies suggested that the vanadium(V) in each complex was reduced to vanadium(IV) during the reactions. All the reactions studied showed first-order kinetics when the concentration of ascorbic acid or glutathione was in large excess and the observed first-order rate constants have a linear relationship with the concentrations of reductant (ascorbic acid or glutathione). Potentiometric results revealed that the most important species in the neutral pH range is $[VO_2(L)_2]^-$ for the V(V) system where L is either ma^- or ema^-. An acid dependence mechanism was proposed from kinetic studies with varying pH and varying maltol concentration. The good fits of the second order rate constant versus pH or the total concentration of maltol, and the good agreement of the constants obtained between fittings, strongly supported the mechanism. Under the same conditions, the reaction rate of $[VO_2(ma)_2]^-$ with glutathione is about 2000 times slower than that of $[VO_2(ma)_2]^-$ with ascorbic acid, but an acid dependence mechanism can also be used to explain the results for the reduction with glutathione. Replacing the methyl group in maltol with an ethyl group has little influence on the reduction rate with ascorbic acid, and the kinetics are the same no matter whether $[VO_2(ma)_2]^-$ or $[VO_2(ema)_2]^-$ is reduced.

Introduction

Although not a common component of enzymes, vanadium, as the vanadate ion, is an essential prosthetic group of some haloperoxidase enzymes,[1] enzymes which are currently being elucidated in great detail.[2] At pharmacological concentrations as an insulin enhancing agent, vanadium and its complexes are attracting increasing attention.[3−5] Both V(V) and V(IV) inorganic salts (vanadate and vanadyl, respectively) as well as some of their complexes are being extensively tested as insulin enhancing drugs (also known as insulin mimics),[5] and it has been found that certain complexes (e.g., BMOV) are more potent and less toxic than the inorganic salts.[6−9] BMOV (bis(maltolato)oxovanadium(IV)) and BEOV (bis(ethylmaltolato)oxovanadium(IV)) are two of the most promising candidates as a substitute or oral complement for insulin for the treatment of diabetes mellitus.[9]

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† Dedicated to Professor K. N. Raymond, UC Berkeley, on the occasion of his 60th birthday.

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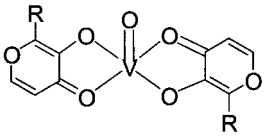


R = CH_3, BMOV, $VO(ma)_2$

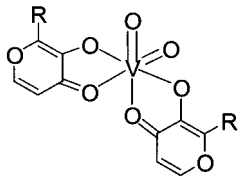
R = C_2H_5, BEOV, $VO(ema)_2$

The redox chemistry of BMOV demonstrates an impressive lability in oxidation and reduction.[10,11] In water or an alcohol, BMOV oxidizes under ambient conditions to form *cis*-$[VO_2(ma)_2]^-$ or *cis*-[VO(OR)$(ma)_2$], respectively, the oxidation kinetics being second-order, a function of the concentrations of both the complex and molecular oxygen.[11] The reaction in water is pH dependent, and it has been shown that both $[VO(ma)_2(H_2O)]$ and $[VO(ma)_2(OH)]^-$ can be oxidized by molecular oxygen.[11]

Although *cis*-$[VO_2(ma)_2]^-$ is not a good insulin enhancing agent,[12] it may be reduced in vivo to form $VO(ma)_2$, which is active. On the other hand, there is precedent for V(V) compounds having insulin-enhancing activity; vanadate and some peroxovanadate complexes have potency.[13] These activities may be related to the reduction of V(V) to V(IV). BMOV is administered to STZ rats in aqueous solution with air excluded, but not rigorously;[6−10] in humans, BEOV is administered in solid form. While it is clear that the in vivo environment is much less aerobic than the in vitro environment, a comprehensive understanding of the $VOL_2/[VO_2L_2]^-$ system is critical to the design of better agents and to improving the efficacy of our existing drug candidates. It has already been shown that BMOV enhances the action of insulin and that *cis*-$[VO_2(ma)_2]^-$ does not;[12] however, the ability of the latter to act as pro-drug for the former may reside in its in vivo redox behavior.

It is well-known that ascorbic acid and glutathione are important to in vivo redox chemistry; the reducing action of ascorbic acid is the basis of its chemical determination.[14] It is known that vanadium(V) can be reduced by ascorbic acid to generate vanadium(IV) at both acidic and physiological pHs.[15−17] In most plant and animal tissues, ascorbate is the only substance which exhibits this reducing action in acid solution.[14] Tissues and bodily fluids contain varying amounts of ascorbic acid, usually below 1−1.2 mg dL^{-1}.[14] Glutathione is required for the action of several enzymes and hormones, such as insulin.[14] Glutathione reductase is thought to function either in insulin degradation or possibly in the formation of the correct disulfide bonds.[14]

In this study, we have determined the kinetic parameters for the reactions of $[VO_2(ma)_2]^-$ and $[VO_2(ema)_2]^-$ with ascorbic acid or glutathione. We hope to delineate the mechanism of the reduction reactions and to find relations between the kinetic parameters and pH, temperature, and ligand concentration. The results would contribute to an understanding of the behavior of vanadium complexes in human tissue and blood.



R = CH_3, $[VO_2(ma)_2]^-$

R = C_2H_5, $[VO_2(ema)_2]^-$

Experimental Section

Materials. Chemicals were reagent grade and used without further purification. $NaVO_3$, ascorbic acid, and glutathione were obtained from Sigma. Maltol (3-hydroxy-2-methyl-4-pyrone, Hma) and ethylmaltol (3-hydroxy-2-ethyl-4-pyrone, Hema) were from Pfizer; stock solutions of $[VO_2(ma)_2]^-$ and $[VO_2(ema)_2]^-$ were freshly prepared every day by dissolving $NaVO_3$ and Hma or Hema (Hma or Hema/$[NaVO_3]$ > 5) in 0.16 M NaCl and 0.1 M Hepes (pH ∼ 7).[10] Standard buffer solutions (pH = 4.00, 7.00), sodium chloride (for controlling the ionic strength of the solution), and sodium hydroxide (for adjusting pH) were purchased from Fisher Scientific, Nepean, Ontario. Hepes (*N*-[2-hydroxyethyl]piperazine *N*′-[2-ethanesulfonic acid] (to buffer the pH of the reaction solution) was from Sigma. Water was deionized (Barnstead D8902 and D8904 cartridges) and distilled (Corning MP-1 Megapure still) before use.

Instrumentation. UV−vis spectra were recorded on a Hewlett-Packard 8453 UV−vis spectrometer, and the temperature was controlled (± 0.02°) by a Fisher Isotemp 1016D thermostat. ^{51}V NMR spectra were recorded on a Varian XL-300 spectrometer (100 kHz spectral width, 12 μs pulse angle, 0.02 s acquisition time) at 78.86 MHz. EPR spectra were recorded on a Bruker ECS-106 EPR spectrometer; the microwave frequency and magnetic field were calibrated with an EIP 625A microwave frequency counter and a Varian E500 gaussmeter, respectively. Potentiometric measurements were carried out with an automatic titration system consisting of a Metrohm 713 pH meter equipped with a Metrohm 6.0233.100 electrode, a model 665 Metrohm Dosimat autoburet, water-jacketed titration vessels, and a Julabo UC circulating bath. Both the pH meter and autoburet were controlled by an IBM-compatible PC, and the titration was controlled by a locally written QBasic program. The electrode was calibrated before each titration by titrating a known amount of aqueous HCl with a known amount of NaOH. A plot of mV(calculated) versus pH gave a working slope and intercept, so that pH could be read as $-\log[H^+]$ directly.

UV Kinetic Measurements. The reductions of $[VO_2(ma)_2]^-$ or $[VO_2(ema)_2]^-$ with ascorbic acid or glutathione were studied in

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aqueous solution (Hepes buffer, $I = 0.16$ M NaCl) at 37 °C. $[VO_2(ma)_2]^-$ or $[VO_2(ema)_2]^-$ stock solutions were freshly prepared every day in 0.16 M NaCl and 0.1 M Hepes. The pH of this solution was usually adjusted to 7.45 with 1 M NaOH. Ascorbic acid or glutathione solutions for each experiment were freshly prepared just before use. Typical steps to prepare solutions are as follows: (1) 0.12 g of Hepes was dissolved in about 9 mL of Ar-saturated aqueous NaCl (0.16 M) and the solution pH adjusted to 7.5; (2) a weighed amount of ascorbic acid or glutathione was added as needed under Ar; (3) the pH was adjusted to 7.45, except in the pH-varying experiments, and the volume adjusted to 10 mL with Ar-saturated water. Ascorbate or glutathione solution (3 mL) was injected into a closed Ar filled cell, and the cell was put into the instrument until the temperature equilibrated to 37 °C. $[VO_2(ma)_2]^-$ solution (0.1 mL) was then injected into the cell, and the absorbance was recorded immediately. The absorbance, over specified time intervals, was recorded for at least 5 half-lives. The absorbance−time data thus obtained were fitted with the HP 8453UV system program (for ascorbic acid) or Sigma plot (for glutathione) on an IBM-compatible computer with a Pentium II processor.

^{51}V NMR Experiments. $[VO_2(ma)_2]^-$ and $[VO_2(ema)_2]^-$ stock solutions, and the ascorbic acid and glutathione solutions, were prepared as were those in the UV experiments (vide supra), except that the concentration of $[VO_2(ma)_2]^-$ or $[VO_2(ema)_2]^-$ was ∼10 times greater. ^{51}V NMR spectra were recorded from time to time after the $[VO_2(ma)_2]^-$ or $[VO_2(ema)_2]^-$ solution was mixed with ascorbic acid or glutathione. The intensity of the signals varied with time, and the data may thus be used to estimate the reaction rate.

EPR Experiments. In a typical run, a solution of $[VO_2(ma)_2]^-$ (0.5 mM) was prepared in 70 mL of Ar-saturated saline solution (0.16 M). This solution (2 mL) was then added to a weighed amount of ascorbic acid or glutathione, and the pH was adjusted to 7.45 with aqueous NaOH or HCl. An EPR spectrum was taken immediately; the elapsed time between the mixing and the beginning of the measurements was 5−15 min. The reduction reaction was followed by monitoring the increase of the peak-to-peak intensity of the first derivative EPR signals. For the same reaction, the intensities of the peaks corresponding to both $M_I = -^7/_2$ and $-^1/_2$ were studied in order to determine the results twice. The cavity of the spectrometer, as well as the glass capillaries used for the EPR studies, exhibited EPR signals. To suppress these extraneous signals from the EPR spectra of the solutions under study, a spectrum of a blank (water in a 20 μL capillary) taken under the same experimental conditions was subtracted. The heights of the resulting first derivative peaks were measured with the peak picking function of the WIN-EPR program.

Results and Discussion

Reaction of $[VO_2(ma)_2]^-$ with Ascorbic Acid. ^{51}V NMR Result. At pH ∼ 7 with maltol in large excess ($[ma]_T/[V(V)]_T > 20/1$), there was only one signal observed in the ^{51}V NMR spectrum (−496 ppm, which is assigned to the vanadium(V) maltol complex $[VO_2(ma)_2]^-$).[10] Signals for vanadate and vanadate oligomers ($V_1 = -560$ ppm, $V_2 = -577$ ppm, $V_4 = -574$ ppm)[10,18] were detected only when maltol was not in large excess. When the reaction of $[VO_2(ma)_2]^-$ with ascorbic acid was followed by ^{51}V NMR, the signal of this V(V) maltol complex decreased with time and disappeared when the reaction finished, a result strongly suggesting that the complex was reduced during the reaction.

EPR Result. A mixture of $[VO_2(ma)_2]^-$ with ascorbic acid eventually showed a typical eight line signal of the vanadyl (oxovanadium(IV)) maltol complex BMOV.[19] The intensity of the resonances increased with time at the beginning and no longer changed after the reaction was finished. The end of the reduction reaction can be determined in terms of the reaction rate constant measured spectrophotometrically under the same conditions. Because time is required to prepare the EPR sample, the latter is not a good method to determine the rate constant for the reaction of $[VO_2(ma)_2]^-$ with ascorbic acid; however, the EPR result clearly shows that the vanadium(IV) maltol complex $VO(ma)_2$ is one of the products of the reduction. For a slower reaction, such as the reduction of $[VO_2(ma)_2]^-$ with glutathione, it is possible to measure the rate constant by EPR (vide infra).

The ^{51}V NMR and EPR results taken together unambiguously delineate that the reaction studied is the reduction of $[VO_2(ma)_2]^-$ by ascorbic acid to form $VO(ma)_2$.

Kinetics of the Reduction of $[VO_2(ma)_2]^-$ with Ascorbic Acid. Rate constants of the reaction were measured by UV spectrophotometry; as the reaction proceeded, the UV spectral absorbance of the reaction mixture between 320 and 350 nm steadily increased. When the ascorbic acid-to-vanadium ratio was in large excess (about 10:1) and the pH of the solution was constant at 7.45, the absorbance−time data pairs can be well-fitted with pseudo-first-order reaction kinetics. Thus, the rate of the reaction is first-order with respect to the total concentration of V(V) (eq 1). A typical plot of absorbance vs time is shown in Figure S1 of the Supporting Information.

$$\text{rate} = \frac{\mathrm{d}[\mathrm{VO(ma)_2}]}{\mathrm{d}t} = k_{\mathrm{obs}}[\mathrm{V(V)}]_{\mathrm{T}} \qquad (1)$$

By varying the ascorbic acid concentration in large excess, a series of observed rate constants was obtained (Table S1). Each constant in Table S1 was determined from an average of the results calculated at 5 different wavelengths and in at least 2 independent kinetic runs. The half-life of the reactions for each ascorbic acid concentration is given in the fourth column, from which it can be seen that most are long enough so that the constants were easily determined. By plotting the observed rate constants k_{obs} versus the concentration of ascorbic acid, a straight line was obtained (Figure 1), implying that the rate is also directly proportional to the concentration of ascorbic acid (eq 2). (In eq 2, AA means

$$\text{rate} = \frac{\mathrm{d}[\mathrm{VO(ma)_2}]}{\mathrm{d}t} = k_{2(\mathrm{AA})}[\mathrm{V(V)}]_{\mathrm{T}}[\mathrm{AA}] \qquad (2)$$

ascorbic acid, and $k_{2(\mathrm{AA})}$ is the second-order rate constant.) The reduction reaction is a second-order reaction, first-order in the concentration of V(V) and first-order in the concentration of ascorbic acid.

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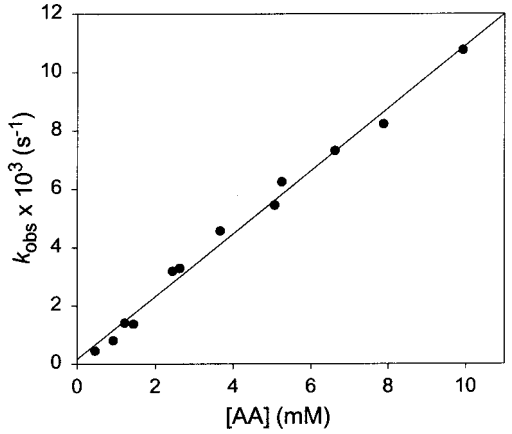

Figure 1. Plot of the observed rate constants k_{obs} vs [AA] with [AA] in large excess (I = 0.16 M NaCl, pH = 7.45, 37 °C, $[V(V)]_T$ = 0.0212 mM, $[ma]_T$ = 0.400 mM).

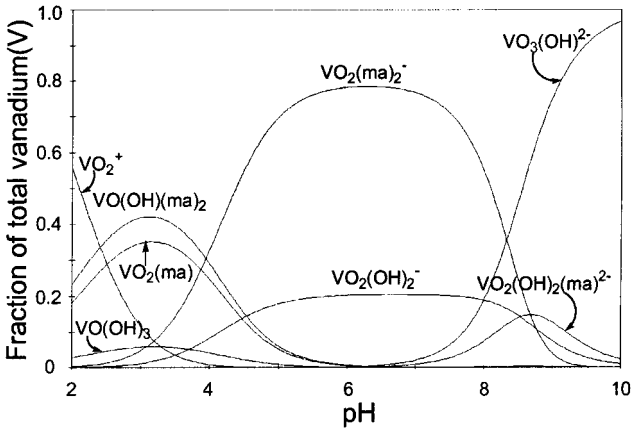

Figure 2. Species distribution diagram of the vanadium(V)−maltol system ($[V(V)]_T/[ma]_T$ = 1/10, $[V(V)]_T$ = 0.0001 M, I = 0.16 M NaCl, 37 °C).

From the slope of the straight line in Figure 1, $k_{2(AA)}$ = 1.08 M^{-1} s^{-1} for the second-order reaction was obtained. In the fifth column of Table S1, the difference between measured k_{obs} and the values calculated according to the straight line are given. It is reassuring to see that in Figure 1 the straight line passes very close to the origin.

$VO(ma)_2$ can be oxidized by molecular oxygen to form $[VO_2(ma)_2]^-$, and the rate of the reaction is first-order in the concentration of $VO(ma)_2$ and first-order in the concentration of O_2 in water.[11] The measured second-order rate constant is 0.21 M^{-1} s^{-1} at pH = 7.25 and 25 °C.[11] If the reaction were to take place under ambient conditions, the calculated rate constant k_{obs} would be 5.7 × 10^{-5} s^{-1}, and the corresponding half-life, 201 min.[11] It is clear that the oxidation of BMOV by O_2 is much slower than the reduction of $[VO_2(ma)_2]^-$ by ascorbic acid. Because air has been excluded in these experiments, the oxidation has no influence on the determination of the reduction reaction rate constant under these conditions.

pH Dependence of the Reduction of $[VO_2(ma)_2]^-$ by Ascorbic Acid. The reduction of $[VO_2(ma)_2]^-$ by ascorbic acid is very pH sensitive. When the concentrations of ascorbic acid, maltol, and $[VO_2(ma)_2]^-$ were varied over a small range, values of k_{obs} were determined for 6.0 ≤ pH ≤ 7.48. When [AA] is in large excess, values of $k_{2(AA)}$ can be calculated according to eq 3 (Tables S2, S3).

$$k_{2(AA)} = k_{obs}/[AA] \quad (3)$$

In Tables S2 and S3, it is shown that acid (H^+) increases the reduction rate dramatically. The pH variation in the experiments is only 1.5, but the highest value of $k_{2(AA)}$ (26.8 at the lowest pH = 6.0) is more than 13 times the lowest value (2.05 at the highest pH = 7.48). This can be explained by including several equilibria in the V(V)−maltol system in solution. These vanadium(V)−maltol equilibria in aqueous solution were studied potentiometrically under the same conditions used for determining the rate constant (I ≤ 0.16 M NaCl, T ≤ 37 °C). The hydrolysis of the metal ion must be accounted for in aqueous solution; when $[V(V)]_T < 10^{-4}$ M, four equilibria must be included in the calculations to determine the stability constants.[20] When a bidentate monoprotic ligand such as the maltol anion, ma^-, is included, the complex-formation reactions shown in eqs 4−8 may occur.

$$VO_2^+ + ma^- \rightleftharpoons [VO_2(ma)]$$

$$K_{110} = [VO_2(ma)]/[ma^-][VO_2^+] \quad (4)$$

$$VO(OH)_3 + ma^- \rightleftharpoons [VO(OH)_3(ma)]^-$$

$$K_{11-1} = [[VO(OH)_3(ma)]^-]/[ma^-][VO(OH)_3] \quad (5)$$

$$[VO_2(OH)_2]^- + ma^- \rightleftharpoons [VO_2(OH)_2(ma)]^{2-}$$

$$K_{11-2} = [[VO_2(OH)_2(ma)]^{2-}]/[ma^-][[VO_2(OH)_2]^-] \quad (6)$$

$$VO_2(ma) + ma^- \rightleftharpoons [VO_2(ma)_2]^-$$

$$K_{120} = [[VO_2(ma)_2]^-]/[ma^-][VO_2(ma)] \quad (7)$$

$$[VO(OH)_3(ma)]^- + ma^- \rightleftharpoons VO(OH)(ma)_2 + 2OH^-$$

$$K_{121} = [VO(OH)(ma)_2][OH^-]^2/[ma^-][[VO(OH)_3(ma)]^-] \quad (8)$$

By taking into account all the hydrolysis constants (the definitions of the hydrolysis equilibria are given in eqs S1−S8 of the Supporting Information) and these complexation equilibria, the potentiometric titration data could be fitted and the relevant stability constants determined. Using the determined stability constants and the hydrolysis constants from the literature,[20] a species distribution diagram can be plotted (Figure 2); it shows that the main V(V) moieties in solution at 6 ≤ pH ≤ 7.5 are $[VO_2(OH)_2]^-$ and $[VO_2(ma)_2]^-$ when the ratio of maltol to V(V) is <20:1. Therefore, for the kinetic studies, the reactions between these two species and ascorbic acid are the most important. Accordingly, an acid-dependent mechanism can be proposed (Scheme 1).

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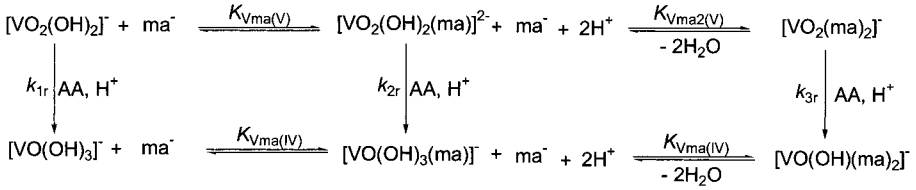
Scheme 1



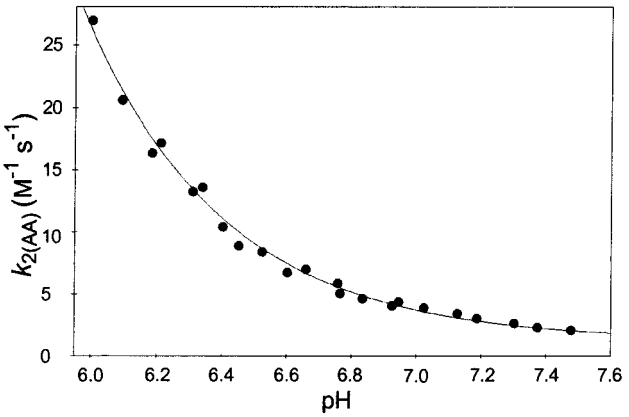


Figure 3. Fit of the experimentally measured rate constants $k_{2(AA)}$ vs pH with eq 12 for the reduction of $[VO_2(ma)_2]^-$ by ascorbic acid ($[V(V)]_T$ = 0.0212 mM, $[V(V)]_T/[ma]_T = 1/20$, I = 0.16 M NaCl, 37 °C).

One can suppose that the chelation reactions (with maltol) are much faster than the reduction reactions in Scheme 1, that is, the equilibria between the vanadium(V) complexes (eqs 9 and 10) are always obtained even when the reduction reactions are taking place. During the titrations of the V(V)−maltol system, when NaOH (0.1 M, 0.2 mL) was added at pH 4, it was found that equilibrium was attained in about 10 s. When maltol alone was titrated, a similar time was needed to reach equilibrium under the same conditions, verifying that the chelation rate between V(V) and maltol is very fast.

$$[VO_2(OH)_2]^- + ma^- \rightleftharpoons [VO_2(OH)_2(ma)]^{2-}$$

$$K_{V(V)ma} = [[VO_2(OH)_2(ma)]^{2-}]/[[VO_2(OH)_2]^-][ma^-] \quad (9)$$

$$[VO_2(OH)_2]^- + 2ma^- + 2H^+ \rightleftharpoons [VO_2(ma)_2]^- + 2H_2O$$

$$K_{V(V)ma2} = [[VO_2(ma)_2]^-]/[[VO_2(OH)_2]^-][ma^-]^2[H^+]^2 \quad (10)$$

According to Scheme 1 and the equilibria, eq 11 can be obtained (details are available in the Supporting Information).

$$k_{2(AA)} = \{k_{1r}[H^+](1 + [H^+]/K_{Hma})^2 + k_{2r}K_{V(V)ma}[ma]_T[H^+](1 + [H^+]/K_{Hma}) + k_{3r}K_{V(V)ma2}[ma]_T^2[H^+]^3\}/\{(1 + [H^+]/K_{Hma})^2 + K_{V(V)ma}[ma]_T(1 + [H^+]/K_{Hma} + K_{V(V)ma2}[ma]_T^2[H^+]^2)\} \quad (11)$$

Equation 11 expresses the relationship between the second-order rate constant ($k_{2(AA)}$) and $[H^+]$; when $[ma]_T$ is held unchanged and only pH is varied, eq 11 can be simplified to eq 12.

$$k_{2(AA)} = \{a(1 + x)^2 + b(1 + x) + cx^2\}x/\{(1 + x)^2 + d(1 + x) + ex^2\} \quad (12)$$

where $x = [H^+]/K_{Hma}$

$$a = k_{1r}K_{Hma}$$

$$b = k_{2r}K_{V(V)ma}[ma]_TK_{Hma}$$

$$c = k_{3r}K_{V(V)ma2}[ma]_T^2K_{Hma}^3$$

$$d = K_{V(V)ma}[ma]_T$$

$$e = K_{V(V)ma2}[ma]_T^2K_{Hma}^2$$

Fitting the experimental data in Table S2 with eq 12 produces a nice fit (Figure 3) and a set of constants $a-e$. From d and e, equilibrium constants log $K_{V(V)ma}$ = 3.4 (eq 9) and log $K_{V(V)ma2}$ = 24.1 (eq 10) were obtained using the known $[ma]_T$ and acidity constant K_{Hma}. These values are the same as those obtained from potentiometric studies and the literature hydrolysis constants,[20] log $K_{V(V)ma}$ = 3.4 and log $K_{V(V)ma2}$ = 24.1, respectively. The excellent fit and agreement of the results from the kinetic study with those from the thermodynamic study strongly support the proposed acid-dependent mechanism.

When $[V(V)]_T$, [AA], and pH are held unchanged but the maltol concentration is varied, $k_{2(AA)}$ should also vary according to eq 11, simplified as eq 13.

$$k_{2(AA)} = (a + bx + cx^2)/(1 + dx + ex^2) \quad (13)$$

where $x = [ma]_T$

$$a = k_{1r}[H^+]$$

$$b = k_{2r}K_{V(V)ma}[H^+]/(1 + [H^+]/K_{Hma})$$

$$c = k_{3r}K_{V(V)ma2}[H^+]^3/(1 + [H^+]/K_{Hma})^2$$

$$d = K_{V(V)ma}/(1 + [H^+]/K_{Hma})$$

$$e = K_{V(V)ma2}[H^+]^2/(1 + [H^+]/K_{Hma})^2$$

A series of experiments varying $[ma]_T$ at pH 7.2 were undertaken to determine a series of rate constants $k_{2(AA)}$. By using the data listed in Table S4 and eq 13, a good fit (Figure 4) and a set of constants were found. From the last two constants (d and e), the equilibrium constants log $K_{V(V)ma}$

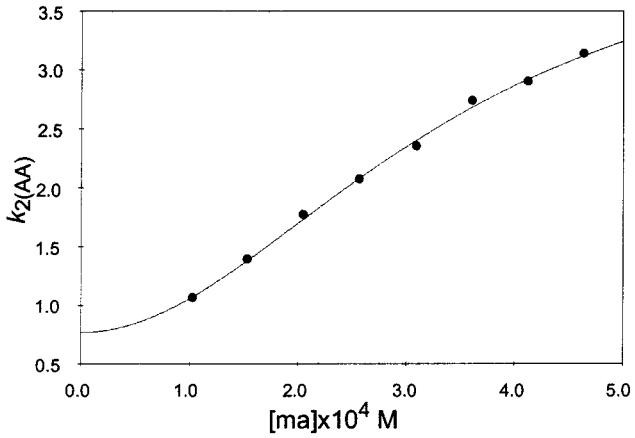


Figure 4. Fit of the experimentally measured rate constants $k_{2(AA)}$ vs $[ma]_T$ with eq 13 (pH = 7.20, I = 0.16 M NaCl, 37 °C, $[V(V)]_T$ = 0.0212 mM, [AA] = 3.664 mM).

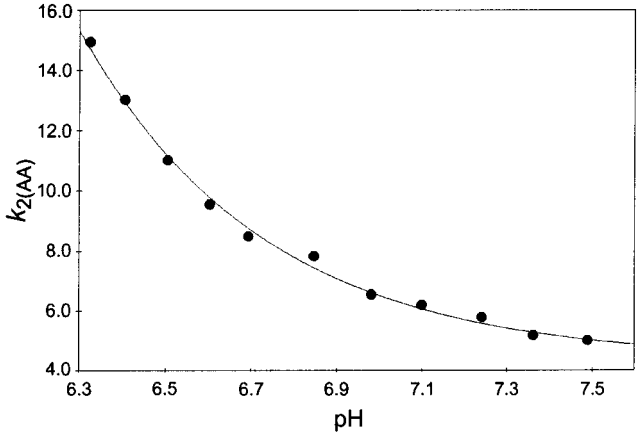


Figure 5. Fit of the experimentally measured rate constants $k_{2(AA)}$ vs pH with eq 14 for the reduction of $[VO_2(ma)_2]^-$ by ascorbic acid ($[V(V)]_T$ = 0.0212 mM, [AA] = 0.43 mM, $[V(V)]_T/[ma]_T$ = 1/25, I = 0.16 M NaCl, 37 °C).

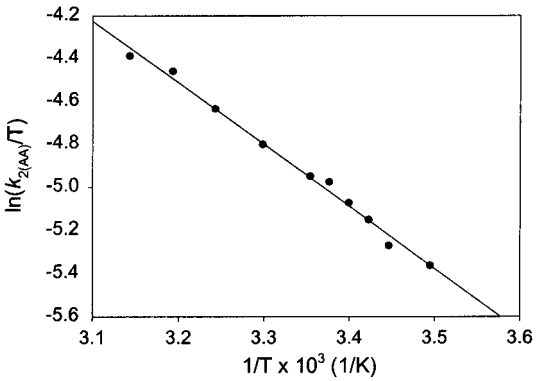


Figure 6. Plot of ln $k_{2(AA)}/T$ vs $1/T$ for the reduction of $[VO_2(ma)_2]^-$ by ascorbic acid (I = 0.16 M NaCl, pH = 7.21, [AA] = 3.7 mM, $[V(V)]_T$ = 0.0212 mM, $[ma]_T$ = 0.410 mM).

(eq 9) and log $K_{V(V)ma2}$ (eq 10) were again obtained, and the results are again close to the results obtained from the $k_{2(AA)}$ versus pH fitting (Table 2). That similar results keep appearing from the different determination methods strongly supports the proposed reaction mechanism.

The rate constants k_{1r}, k_{2r}, and k_{3r} from the $k_{2(AA)}$ versus pH fitting and the $k_{2(AA)}$ versus $[ma]_T$ fitting are given in Table 2. The agreement of all the constants from the two fittings is really not bad for such a complicated system; the order of the three constants is the same no matter the fitting from which they are generated, that is, $k_{3r} > k_{1r} > k_{2r}$. This result implies that the reduction of the bis maltol complex is the main path for the reduction of vanadium(V) in this system. This is especially true when the ratio $[ma]_T/[V(V)]_T$ is high and the pH is low. The calculation suggests that, even at $[ma]_T/[V(V)]_T$ = 10/1 and pH = 7.45, the reduction of $[VO_2(ma)_2]^-$ is still four times faster than the reduction of $[VO_2(OH)_2]^-$. Only when $[ma]_T/[V(V)]_T$ is as low as 5/1 do the two reactions have almost equal rates. Using the values given in Table 2, however, it was found that although the second term in eq 11 can be omitted when $[ma]_T/[V(V)]_T >$ 20/1, the first term in eq 11 can never be omitted even when $[ma]_T/[V(V)]_T$ = 100/1. Hence, under the condition $[ma]_T/[V(V)]_T$ = 25/1, the $k_{2(AA)}$ values obtained at different pHs (Table S3) were well fit with eq 14 (Figure 5).

$$k_{2(AA)} = \{k_{1r}[H^+](1 + [H^+]/K_{Hma})^2 + k_{3r}K_{V(V)ma2}[ma]_T^2[H^+]^3\}/\{(1 + [H^+]/K_{Hma})^2 + K_{V(V)ma2}[ma]_T^2[H^+]^2\} \quad (14)$$

The result implies that when the $[ma]_T/[V(V)]_T$ ratio is large enough, only two V(V) species ($[VO_2(OH)_2]^-$ and $[VO_2(ma)_2]^-$) contribute to the reduction rate.

Temperature Dependence of the Reduction of $[VO_2(ma)_2]^-$ with Ascorbic Acid. The temperature dependence of the reaction of $[VO_2(ma)_2]^-$ with ascorbic acid was studied at constant maltol and ascorbic acid concentrations, and pH = 7.21. Varying upward, 13 °C ≤ T ≤ 45 °C, the measured $k_{2(AA)}$ values increased from 1.34 to 3.95 (Table S5). Plotting ln $k_{2(AA)}/T$ versus $1/T$ (T, K) produces a straight line (Figure 6).

The result seems surprising, initially, because eq 11 suggests that the situation should not be so simple. However, in checking eq 11 carefully, it was found that if the activation enthalpies ($\Delta H^{\ddagger}$) for all three reduction reactions are similar (with T = temperature (K); R = 8.314 (J mol^{-1} K^{-1}); A_1, A_2, and A_3 are temperature independent factors), then

$$k_{2(AA)} = [Te^{-\Delta H^{\ddagger}/RT}[H^+]\{A_1(1 + [H^+]/K_{Hma})^2 + A_2(1 + [H^+]/K_{Hma})K_{V(V)ma}[ma^-]_T + A_3K_{V(V)ma2}[ma^-]_T^2[H^+]^2\}/[\{(1 + [H^+]/K_{Hma})^2 + (1 + [H^+]/K_{Hma})K_{V(V)ma}[ma^-]_T + K_{V(V)ma2}[ma^-]_T^2[H^+]^2\}] \quad (15)$$

In eq 15, the upper terms in brackets and the lower terms in brackets are very similar, and the only differences are the three temperature independent factors. Actually, it was found that the quotient of the terms in brackets is independent of temperature. Therefore, from eq 15, it can be deduced that there is a linear relationship between ln $k_{2(AA)}/T$ and $1/T$; this agrees with our experimental results.

Kinetics of the Reaction of $[VO_2(ma)_2]^-$ with Glutathione. The reduction of $[VO_2(ma)_2]^-$ by glutathione is much slower than the reduction of $[VO_2(ma)_2]^-$ by ascorbic acid; hence, the reaction is accessible on the EPR time scale, and the rate constant (Table S6) could be determined by

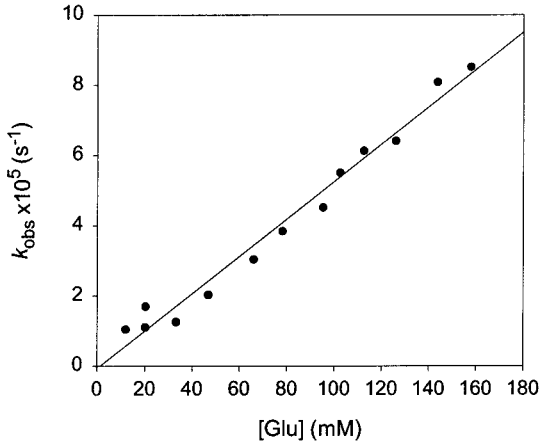


Figure 7. Plot of the observed rate constants k_{obs} vs the concentration of glutathione for the reduction of $[VO_2(ma)_2]^-$ by glutathione (I = 0.16 M NaCl, 37 °C, pH = 7.45, $[V(V)]_T$ = 0.0529 mM, $[ma]_T$ = 2.054 mM).

monitoring EPR spectral intensity data at different times. EPR spectra proved that the reaction product is also BMOV, and when glutathione was in large excess the reaction has typical first-order kinetics (Figure S2).

The rate constant was also measured by following the reaction in the UV spectrum, and similarly, a first-order reaction kinetics was apparent when the glutathione concentration was in excess versus the $[VO_2(ma)_2]^-$ concentration (Figure S3). The observed first-order rate constants determined at different glutathione concentrations are listed in Table S6, in which each value is from the average of 4 constants measured at different wavelengths. By plotting the observed rate constants, which were measured when the glutathione concentration was in large excess, against the concentration of glutathione, a straight line was obtained (Figure 7). Although the fit in this case is worse than that for ascorbic acid (Figure 1), the result proves that the reaction is also first-order in the concentration of glutathione. From the linear regression, the second-order rate constant $k_{2(glu)} = 5.30 \times 10^{-4}$ M^{-1} s^{-1} was obtained. Upon comparison with the corresponding result for ascorbic acid ($k_{2(AA)} = 1.08$ M^{-1} s^{-1}), it can be seen that the rate of the reduction of $[VO_2(ma)_2]^-$ by ascorbic acid is about 2000 times faster than that by glutathione.

pH Dependence of the Reduction of $[VO_2(ma)_2]^-$ by Glutathione. The rate of reduction of $[VO_2(ma)_2]^-$ by glutathione is also very sensitive to pH; when the glutathione concentrations were closely varied, the rate constants measured versus pH are summarized in Table S7. It is reasonable to suppose that the acid dependence reaction mechanism is similar to that in the $[VO_2(ma)_2]^-$−ascorbic acid system, according to the above discussion; that is, eq 11 still holds for glutathione as the reducing agent. To prove the assumption, the $k_{2(glu)}$−pH data listed in Table S7 with eq 12 (Figure 8) and the constants calculated are given in the last row of Table 2. Despite the different pH ranges used, it is clear that the fits are quite good and that the constants log $K_{V(V)ma}$ (eq 9) and log $K_{V(V)ma2}$ (eq 10) are very close to the corresponding constants determined according to the pH dependence study of the $[VO_2(ma)_2]^-$−ascorbic acid system.

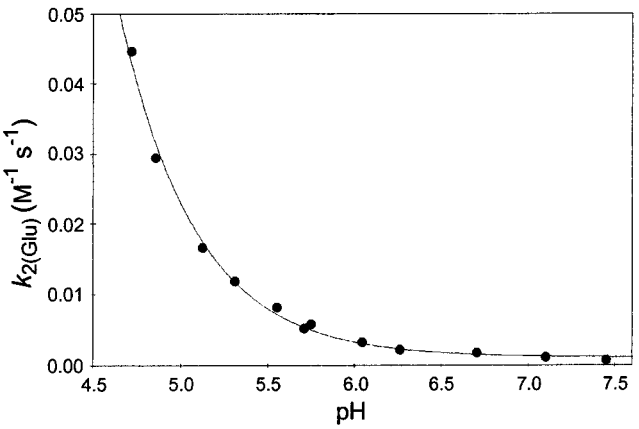


Figure 8. Fit of the experimentally measured rate constants $k_{2(glu)}$ vs pH with eq 12 for the reduction of $[VO_2(ma)_2]^-$ by glutathione ($[V(V)]_T$ = 0.0529 mM, $[V(V)]_T/[ma]_T$ = 1/38, I = 0.16 M NaCl, 37 °C).

We have also tried to vary only the concentration of maltol to measure the second-order rate constant for the reduction of $[VO_2(ma)_2]^-$ by glutathione (Table S8). It is clear that $k_{2(glu)}$ is not so sensitive to the concentration of maltol; $[ma]_T$ varies from 0.256 to 2.122 mM, an increase of 8.3 times; $k_{2(glu)}$ changes from 4.17×10^{-3} to 5.79×10^{-3} M^{-1}s^{-1}, an increase of only 39%. When ascorbic acid was the reducing agent, a 4.5 times increase of maltol concentration tripled $k_{2(AA)}$. As was done in the $[VO_2(ma)_2]^-$−ascorbic acid system, the $k_{2(glu)}$ values in Table S8 were also fit against the total concentration of maltol (Figure S4). Because of the slow rate and the small difference between the rate constants, the fit is not as good as is that for the ascorbic acid system. The constants obtained from the fit are also not in good agreement with the values obtained from the variable pH fitting (Table 2); however, the result is still reasonable and again supports the acid dependence mechanism.

Recently, the coordination of glutathione to VO^{2+} in aqueous solution has been studied.[21,22] It is known that oxidized glutathione is a more efficient VO^{2+} binder than is reduced glutathione.[23,24] However, according to eq 11, the coordination of ligands to VO^{2+} should have no influence on the reduction rate because the rates of the coordination reactions are much faster than those of the reductions, especially for glutathione as the reducing agent.

Kinetics of the Reduction of $[VO_2(ema)_2]^-$ by Ascorbic Acid. The reaction of the vanadium(V) ethylmaltol complex (*cis*-$[VO_2(ema)_2]^-$) by ascorbic acid was also studied by UV spectrophotometry. It is also a pseudo-first-order reaction when the ascorbic acid concentration is in large excess. The pseudo-first-order rate constants measured at pH = 7.45, T = 37 °C, with different ascorbic acid concentrations, are summarized in Table S9. By plotting the rate constants versus the concentration of ascorbic acid, a linear relationship is again found (Figure S5), and the corresponding second-order rate constant was determined to be $k_{2(AA)} = 0.606$ M^{-1} s^{-1}.

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Table 1. Stability Constants of the Complexes Formed between V(V) and Maltol or Ethylmaltol Determined Potentiometrically ($I = 0.16$ M NaCl, 37 °C)[a]

ligand	maltol	ethylmaltol
pK_a	8.67 ± 0.03	8.72 ± 0.03
log K_{110}	9.21 ± 0.15	9.22 ± 0.15
log K_{120}	7.87 ± 0.12	7.84 ± 0.07
log K_{121}	7.3 ± 0.2	7.46 ± 0.08
log K_{11-2}	3.4 ± 0.2	3.40 ± 0.07
log K_{11-1}	4.1 ± 0.2	4.09 ± 0.11

[a] The error limits given are 3σ.

Table 2. Kinetic and Thermodynamic Parameters Obtained by Different Methods (HEPES buffer, $I = 0.16$ M NaCl, 37 °C)

method	log k_{1r} (M^{-2} s^{-1})	log k_{2r} (M^{-2} s^{-1})	log k_{3r} (M^{-2} s^{-1})	log $K_{V(V)ma}$	log $K_{V(V)ma2}$
potentiometry				3.4 ± 0.2	24.1 ± 0.3
$k_{2(AA)}$ vs pH	6.9	6.6	7.6	3.4	24.1
$k_{2(AA)}$ vs $[ma]_T$	7.1	6.7	7.8	3.1	24.3
$k_{2(glu)}$ vs pH	4.3	3.5	3.1	3.4	24.1
$k_{2(glu)}$ vs $[ma]_T$	4.0	3.3	4.0	3.5	23.5

Comparing this result with the value obtained for $[VO_2(ma)_2]^-$ ($k_{2(AA)} = 1.08$ M^{-1} s^{-1}), it can be seen that the methyl for ethyl substitution has some small effect on the reduction rate with ascorbic acid. This is different from the stability constants for $[VO_2(ma)_2]^-$ and $[VO_2(ema)_2]^-$ which are essentially the same (Table 1). This result is unsurprising because the replacement of a methyl group in maltol with an ethyl group should not make a significant change in the basicity of the ligand. The larger ethyl group could, however, provide a greater steric inhibition to the approaching ascorbic acid, thus decreasing the rate constant. It is reasonable to think that all other properties of the reduction of $[VO_2(ema)_2]^-$ by ascorbic acid should be very similar to those for the reduction of $[VO_2(ma)_2]^-$ by ascorbic acid.

Biological Implications. The present work tends to corroborate earlier studies showing a transitory depression in glutathione concentration following acute administration of V(V),[25] or an elevation of tissue glutathione levels following chronic administration.[26,27] Results of this study demonstrate that reduction of V(V) by glutathione at physiologically relevant pHs will be far slower than with ascorbate, another highly prevalent low molecular weight cellular reductant. This situation is analogous to the in vitro observation of Cr(VI) reduction by ascorbate in preference to glutathione[28] and by the well-known increased bioavailability of Fe(III) in vivo in the presence of excess ascorbate in the diet.[29]

As mentioned before, the tissues and body fluids contain usually 10−12 mg L^{-1} of ascorbic acid;[14] this corresponds to a concentration of about 0.057−0.068 mM. Pharmacokinetic studies for BEOV[30] showed that after acute oral administration of BEOV (oral gavage dose 0.030 mmol kg^{-1}) there is a peak of [V(IV)] in plasma or blood after 1 h. The corres ponding concentration of vanadium at the peak is about 0.04 mM.[30] If a similar dose of $[VO_2(ma)_2]^-$ were given, at the peak concentration the total maltol concentration would be 0.08 mM. At pH = 7.45 and $[ma]_T = 0.08$ mM, the calculated $k_{2(AA)}$ value is 0.40 M^{-1} s^{-1}. Combining this value with an average in vivo concentration of ascorbic acid (0.062 mM), the calculated half-life of $[VO_2(ma)_2]^-$ in vivo should be 7.7 h. This discussion suggests that if only ascorbic acid is considered as the relevant biological reductant, $[VO_2(ma)_2]^-$ should be quite stable in tissues or blood. At a stomach pH of 2, however, with the concentration of ascorbic acid at 0.062 mM, the corresponding half-life of $[VO_2(ma)_2]^-$ would be 0.1 s.

It is also clear from the present work that the relatively slow reduction of $[VO_2(ma)_2]^-$ ($t_{1/2} = 7.7$ h) and $[VO_2(ema)_2]^-$ even in the presence of the faster-acting ascorbate may underlie failure of the former compound to evince any glucose-lowering effect in an in vivo acute oral or ip screening test.[12] According to recent pharmacokinetic studies showing rapid disappearance of vanadium and ethylmaltol from plasma following an acute oral administration of $VO(ema)_2$,[30] the half-life of $[VO_2(ema)_2]^-$ would far exceed that of expected maximal glucose-lowering, at pH 7.4, as administered. In a chronic feeding situation, the lower pH of the stomach could be expected to speed reduction, thus potentially rendering a V(V) pro-drug (such as $[VO_2(dipic)]^-$ [13] or the herein studied $[VO_2(ema)_2]^-$ and $[VO_2(ma)_2]^-$) effective as a glucose-lowering agent, following ascorbate reduction to V(IV).

Conclusions

The reduction of $[VO_2(ma)_2]^-$ by ascorbic acid is a second-order reaction, first-order with respect to the total concentration of vanadium(V) and first-order with respect to the total concentration of ascorbic acid. The second-order rate constant $k_{2(AA)}$ is very sensitive to the pH of the solution, and an acid dependence mechanism has been deduced from both the kinetic and thermodynamic results. The good fits of $k_{2(AA)}$ versus pH and of $k_{2(AA)}$ versus the total concentration of maltol, as well as the good agreement of the constants obtained from the different fittings, strongly support the mechanism. Although the reduction of $[VO_2(ma)_2]^-$ by glutathione is much slower than that by ascorbic acid, the studies show that both reactions have similar properties and mechanism.

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Supporting Information Available: Additional information, tables, and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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