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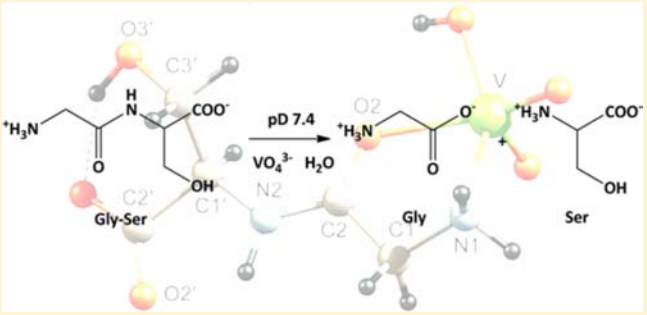
Hydrolytic Activity of Vanadate toward Serine-Containing Peptides Studied by Kinetic Experiments and DFT Theory

Phuong Hien Ho,[†] Tzvetan Mihaylov,[†] Kristine Pierloot,[†] and Tatjana N. Parac-Vogt*,[†]

[†]Department of Chemistry, KU Leuven, Celestijnenlaan 200F, B-3001, Leuven, Belgium

Supporting Information

ABSTRACT: Hydrolysis of dipeptides glycylserine (Gly-Ser), leucylserine (Leu-Ser), histidylserine (His-Ser), glycylalanine (Gly-Ala), and serylglycine (Ser-Gly) was examined in vanadate solutions by means of ^{1}H, ^{13}C, and ^{51}V NMR spectroscopy. In the presence of a mixture of oxovanadates, the hydrolysis of the peptide bond in Gly-Ser proceeds under the physiological pH and temperature (37 °C, pD 7.4) with a rate constant of 8.9×10^{-8} s^{-1}. NMR and EPR spectra did not show evidence for the formation of paramagnetic species, excluding the possibility of V(V) reduction to V(IV) and indicating that the cleavage of the peptide bond is purely hydrolytic. The pD dependence of k_{obs} exhibits a bell-shaped profile, with the fastest hydrolysis observed at pD 7.4. Combined ^{1}H, ^{13}C, and ^{51}V NMR experiments revealed formation of three complexes between Gly-Ser and vanadate, of which only one complex, designated Complex 2, formed via coordination of amide oxygen and amino nitrogen to vanadate, is proposed to be hydrolytically active. Kinetic experiments at pD 7.4 performed by using a fixed amount of Gly-Ser and increasing amounts of Na_3VO_4 allowed calculation of the formation constant for the Gly-Ser/VO_4^{3-} complex (K_f = 16.1 M^{-1}). The structure of the hydrolytically active Complex 2 is suggested also on the basis of DFT calculations. The energy difference between Complex 2 and the major complex detected in the reaction mixture, Complex 1, is calculated to be 7.1 kcal/mol in favor of the latter. The analysis of the molecular properties of Gly-Ser and their change upon different modes of coordination to the vanadate pointed out that only in Complex 2 the amide carbon is suitable for attack by the hydroxyl group in the Ser side chain, which acts as an effective nucleophile. The origin of the hydrolytic activity of vanadate is most likely a combination of the polarization of amide oxygen in Gly-Ser due to the binding to vanadate, followed by the intramolecular attack of the Ser hydroxyl group.



INTRODUCTION

Vanadium is considered to be an essential element which has a beneficial effect at low concentrations but becomes toxic when present in higher concentrations.[1] The biological role of vanadium has been widely investigated; however, despite many studies, its exact role is not yet fully understood. The recent medicinal applications of vanadium are reflected in exploring the use of structure−activity relationships of antidiabetes vanadium complexes, development of vanadate compound as antibacterial and antitumor agents, and investigation of osteogenic properties of several vanadium compounds.[2,3] Several excellent reviews on the biological and medicinal importance of vanadium and its compounds have been published.[4]

Vanadate, ($H_2VO_4^-$/HVO_4^{2-}) which is the most commonly occurring form of vanadium(V), has very complex solution chemistry and tendency to form different polyoxoanions at physiological pH, which complicates the thorough understanding of its biological role. The monomoric vanadate (V_1) is a strong inhibitor of myosin and several enzymes, including ATPases, ribonucleases, and phosphatases. Due to its structural analogy to phosphate, the monomeric form has been very extensively studied; however, a number of studies have emerged during the last two decades revealing that oligomeric forms, such as tetrameric $[V_4O_{12}]^{4-}$ (V_4), pentameric $[V_5O_{15}]^{5-}$ (V_5), and decameric $[V_{10}O_{28}]^{6-}$ (V_{10}) oxovanadates, also possess important biological functions.[5,6] The interconversion between different oligomeric species is possible; however, different biological activity was observed depending on which oligomer is initially present in the solution.[6−10] Despite a number of studies which point toward the biological importance of vanadate and its polyoxo forms, the molecular basis for this activity remains largely unexplored. While it has been mainly assumed that the mode of action of oxo-vanadates mostly depends on their structural features such as the charge and the size of the species,[11] the potential reactivity of oxo-vanadates toward biologically relevant substrates has been scarcely explored.

In our quest in understanding the biological role of oxovanadates, we recently focused our attention on probing their reactivity toward biologically relevant molecules and their

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model systems. Our studies have shown that oxovanadates efficiently hydrolyze a series of carboxyesters, as well as a phosphoester bond in commonly used RNA and DNA model substrates such as 2-hydroypropyl-4-nitrophenyl phosphate (HPNP), 4-nitrophenyl phosphate (NPP), and bis-4-nitrophenyl phosphate (BNPP).[12−14] The kinetic studies in which the rate profile was compared with the concentration profile of different oxovanadates present in solution, combined with different spectroscopic techniques, allowed for the identification of hydrolytically active species. Interestingly, depending on the type of substrate, different oxovanadates were implicated as kinetically active. While in the case of NPP and BNPP hydrolysis V_{10} was identified as the reactive oxoanion, in the transesterification of HPNP the V_4 and V_5 were implicated as the reactive complexes.[12,13] The origin of the hydrolytic activity of polyoxovanadates was attributed to their high internal lability and their known ability to react with phosphate derivatives.

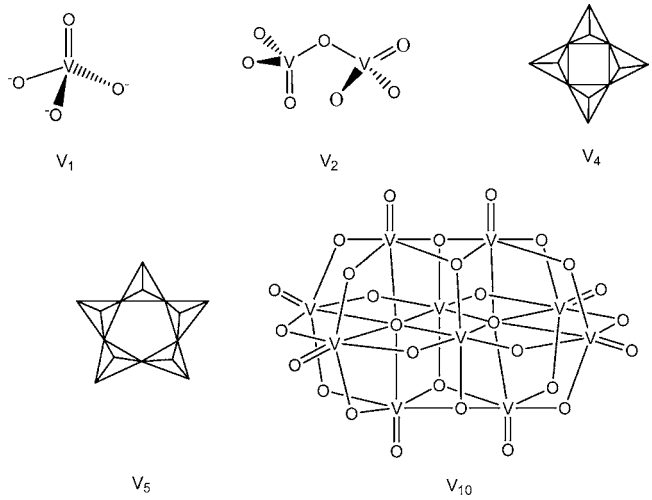
These initial studies encouraged us to further explore the reactivity of oxovanadates toward a range of different peptides, another family of molecules with an important biological relevance. The photooxidative cleavage of proteins in the presence of a mixture of oxovanadates has been previously reported.[15,16] The photocleavage involving the free radical addition to the carbon atom in the peptide bond has been proposed; however, the exact molecular mechanism has not been elucidated. Interactions between vanadate and dipeptides have been also previously studied, mostly by the means of ^{51}V NMR spectroscopy.[17−21] For peptides with noncoordinating side chains a 6-fold coordination geometry around vanadium has been suggested at physiological pH, with coordination of the peptide occurring via the terminal amino and the carboxylate groups as well as via the nitrogen in the peptide bond. However, for the peptides containing a hydroxyl group in the side chain, coordination of this group has been also suggested, particularly upon the increase of pH. These findings were also confirmed by density-functional study and molecular dynamics calculations methods.[22,23] While these studies offer important additional insight into the mode of vanadium binding sites in proteins, they do not report on the potential reactivity of vanadate toward the peptide bond.

In this study we explore the reactivity of oxovanadates toward a range of peptides and, to the best of our knowledge, report on the first example of a peptide bond hydrolysis promoted by a vanadate anion under physiological pH. It is to be noted that the remarkable inertness of peptide bond, with an estimated half-life of up to 600 years under physiological pH and temperature, makes its hydrolysis a challenging task.[24] A number of metal complexes including zinc(II),[25] copper(II),[26] nickel(II),[27] palladium(II),[28] cerium(IV),[29] zirconium(IV),[30] and vanadium(IV)[31] have been found to be effective at promoting hydrolysis of unactivated amide bonds in peptides and proteins. Besides the basic requirement that the complex promotes amide bond cleavage, the selectivity of the cleavage remains a big challenge, and very few complexes were reported to promote a residue selective hydrolysis of peptides. Pt(II) and Pd(II) complexes were shown to promote selective hydrolysis at cysteine, methionine, and histidine residues,[32] while molybdocene dichloride complex was used for the hydrolysis of cysteine-containing peptides.[33] Selective hydrolysis of serine-containing peptides by zinc(II) salts has been reported by the group of Komiyama,[25] and more recently Ni(II) ions have been also used to selectively hydrolyze X-Ser sequence.[27] Despite the wide structural variety of complexes that were active toward hydrolysis of a peptide bond, most of them share some basic requirements such as overall positive charge and the presence of coordinated water molecule, allowing for the metal coordinated water molecules to act as an intramolecular general acid catalyst or for the nucleophile activation of coordinated water or hydroxide ligand. However, in our recent report we demonstrated that despite its negative charge and lack of coordinated water or hydroxide, the molybdate anion efficiently hydrolyzes the peptide bond in serine containing peptides.[34] Considering the structural analogy between molybdate and vanadate anions, and the important role of vanadium in various biochemical processes such as the regulation of enzymatic phosphorylations, the inhibition of the Na, K ATPase, the chlorophylly synthesis[4b] in this study we examine its reactivity toward a series of peptides and give a full account on the mechanism of this novel reaction by combining kinetic experiments and DFT-based molecular modeling.

RESULTS AND DISCUSSIONS

Hydrolysis of Peptides by Vanadium(V) Oxoanions. The reaction between vanadate(V) oxyanions with a range of peptides was initially examined in aqueous solutions at pD 4.4 and pD 7.4. Under these pD conditions a mixture containing different amounts of V_1, V_2, V_4, V_5, and V_{10} oxoanions (shown in Scheme 1) is present in solution.

Scheme 1. Structures of Mono-, Di-, Tetra-, Penta-, and Decavanadate



The degree of peptide bond hydrolysis was determined after 140 h by integration of the proton NMR resonances of the peptide and free amino acid products. The results (Table S1) indicate that at pD 7.4 the conversion ranged from 3% for Gly-Phe to 52% for Gly-Ser. It is important to note that all reactions go to completion; however, for the sake of comparison the yield of hydrolysis was determined after 140 h for all the examined peptides. Among all the peptides, those with a X-Ser and X-Thr sequence were most readily hydrolyzed (Table 1). These peptides have a serine or threonine residue at the C-terminus, indicating the importance of the hydroxyl group in the side chain for efficient hydrolysis.

Based on the results presented in Table 1 and Table S1, which show the fastest hydrolysis for serine-containing peptides, the further work was focused on the series of dipeptides containing serine residues, such as glycylserine (Gly-

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Table 1. Extent of Peptide Bond Hydrolysis in Selected Dipeptides (2.0 mM) Measured after 140 h in the Absence and in the Presence of 25.0 mM Na_3VO_4 (pD 7.4 and 4.4) at 60 °C

		conversion (%) with Na_3VO_4		conversion (%) without Na_3VO_4	
no.	dipeptide	pD 7.4	pD 4.4	pD 7.4	pD 4.4
1	Gly-Ser	52	5	7	3
2	His-Ser	40	25	10	5
3	Leu-Ser	39	12	6	2
4	Ser-Gly	3	6.0	0	0
5	Gly-Ala	7	8	0	0
6	Gly-Thr	13	15	6	2
7	Gly-Ser-Phe	16	4	0	0

Ser), leucylserine (Leu-Ser), histidylserine (His-Ser), and serylglycine (Ser-Gly). For comparison, glycylalanine (Gly-Ala) was studied as well (Scheme 2).

The Hydrolysis of Gly-Ser by Vanadate(V) Oxyanions. The reaction between Gly-Ser and vanadate(V) oxyanions was performed by adding 2.0 mM of Gly-Ser to a 20.0 mM solution of Na_3VO_4 at pD 7.4 and incubating the sample at 60 °C. ^{1}H NMR spectra measured at different time increments showed a gradual intensity decrease of the signal corresponding to the CH_2 protons of the glycyl residue in Gly-Ser at 3.89 ppm and the appearance of a CH_2 resonance at 3.50 ppm attributed to glycine. The identity of the latter peak was confirmed by spiking experiments in which a solution of free glycine was added to the reaction mixture, resulting in an intensity increase of the resonance at 3.50 ppm. As can be seen in Figure S1, the intensity of all other resonances, at 3.79−3.95 ppm (CH_2 of serine residue) and 4.35 ppm (CH of serine residue), that were attributed to Gly-Ser also decreased during the course of reaction, while the appearance of free serine (regions 3.75−3.83 and 3.86−4.00 ppm) was simultaneously observed. These data unambiguously indicate that the cleavage of the peptide bond in Gly-Ser occurred. Interestingly, during the course of hydrolytic reaction, the presence of a small amount of cyclic Gly-Ser (cGly-Ser) was also observed in the NMR spectrum, characterized by a singlet at 3.55 ppm and multiplet peaks in the region of 4.11−4.15 ppm, indicating that vanadate also promotes cyclization of Gly-Ser. The cyclization to cGly-Ser by vanadate is an irreversible process as no hydrolysis of cGly-Ser into Gly and Ser was detected. Therefore, the full hydrolysis of Gly-Ser resulted in a mixture containing 90% of Gly and Ser and 10% of cGly-Ser. Based on the integration of the proton NMR resonances at different reaction times the hydrolysis rate constant of Gly-Ser was calculated: $k_{obs} = 1.3 \times 10^{-6}$ s^{-1} at 60 °C and pD 7.4 (Figure 1). For comparison, while in the presence of vanadate complete hydrolysis of Gly-Ser was observed, only 15% of Gly-Ser was hydrolyzed in the absence of vanadate under the same conditions. It is worth mentioning that the reaction also occurred under physiological pH and temperature (37 °C, pD 7.4) with a rate constant of 8.9×10^{-8} s^{-1}, under which spontaneous hydrolysis of Gly-Ser was extremely slow.

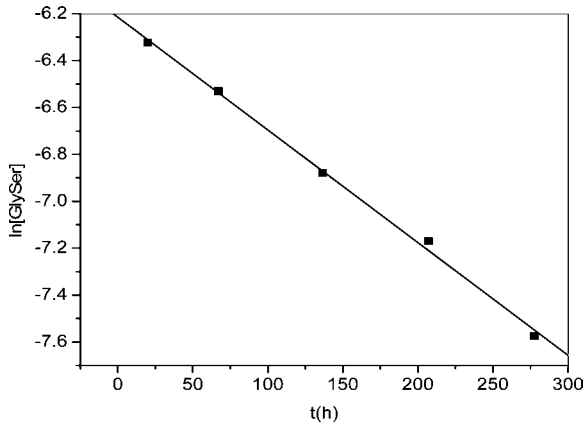
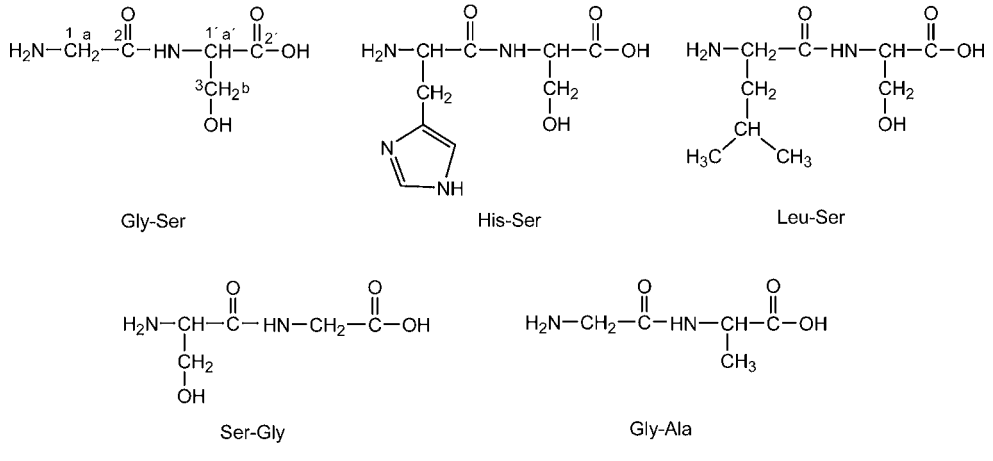


Figure 1. Kinetic profile for the hydrolysis of Gly-Ser in the presence of Na_3VO_4 in a D_2O solution at pD 7.4 and 60 °C.

Importantly, NMR and EPR spectra did not show any evidence of paramagnetic species, which excludes the possibility of oxidative cleavage and the reduction of V(V) to V(IV). These results suggest that the reaction is purely hydrolytic in nature and can be presented as shown in Scheme 3.

Due to the importance of pH in the hydrolysis of peptide substrates, as well as in the speciation of different oxovanadate anions, the effect of pD on the hydrolysis rate of Gly-Ser was examined in detail. For comparison, the hydrolysis of Gly-Ser was also followed in the absence of vanadate in the pD range from 5.2 to 10.0 (Table S2), and the significant rate acceleration clearly showed that the Gly-Ser is hydrolyzed due to the presence of oxovanadates.

Scheme 2. Structure of Peptides Used in This Study



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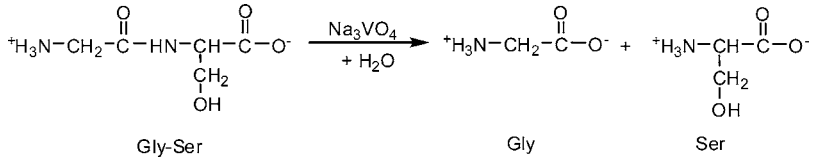
Scheme 3. Schematic Representation of Gly-Ser Hydrolysis in the Presence of Vanadate



At pD 7.4 the monomeric V_1 coexists in equilibrium with V_2, V_4, and V_5. Upon acidification the formation of the decavanadate V_{10} and its protonated forms takes place in solution. The data shown in Figure 2 indicated that the hydrolysis was very slow in acidic media and that the rate of Gly-Ser hydrolysis at pD 5.0 (k_{obs}= 4.6 × 10^{-7} s^{-1}) in the presence of decavanadate was very similar to the spontaneous hydrolysis of Gly-Ser (k_{obs} = 2.8 × 10^{-7} s^{-1}) indicating that V_{10} was not active toward Gly-Ser hydrolysis. The slow hydrolysis of other dipeptides at pD 4.4 (Table 1 and Table S1) in the presence of vanadate also suggest that V_{10} is a hydrolytically inactive species. The pD dependence of k_{obs} exhibits a bell-shaped profile, which fastest cleavage observed at pD 7.4. The data points were fitted to a Michaelis function (eq 1) describing a bell-shaped dependence of the rate constant on pD, where k_{obs} is the measured kinetic constant and h = 10^{-pD} and k, K_1, and K_2 are parameters to be estimated[35]

$$k_{obs} = k/(1 + h/K_1 + K_2/h) \quad (1)$$

The fastest Gly-Ser hydrolysis occurs at neutral pD where a mixture of V_1, V_2, V_4, and V_5 is present in solution. The similarity in the shape of the kinetic profile and the fraction distribution of V_2, V_4, and V_5 would indicate that these oxoanions are hydrolytically active toward Gly-Ser hydrolysis. The various forms of vanadium(V) oxo species were determined by means of ^{51}V NMR spectroscopy. However, previous studies examining the equilibrium of solutions containing a mixture of oxovanadate species revealed that these oxovanadates interconvert on the millisecond time scale, according to the equations below[17]

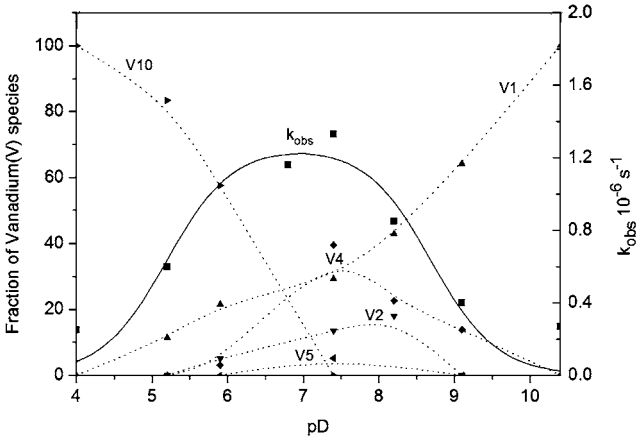


Figure 2. The pD dependence of k_{obs} (■, solid line) for the cleavage of 2.0 mM of Gly-Ser in the presence of 20.0 mM of Na_3VO_4. The fractions of different oxovanadium species have been added for comparison.

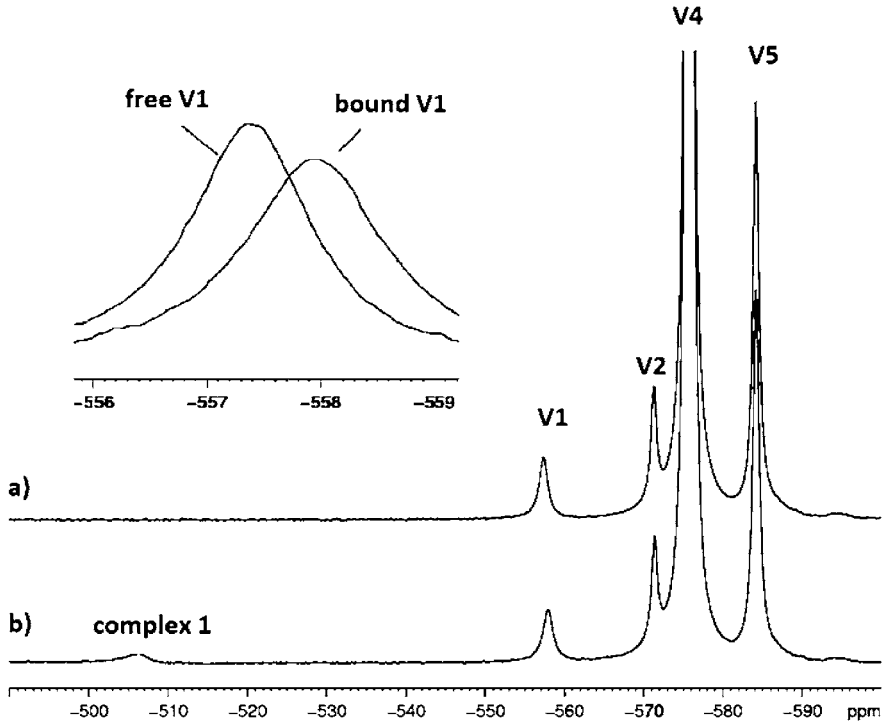


Figure 3. ^{51}V NMR spectra of solution containing 25 mM Na_3VO_4 recorded in the absence (a) and the presence (b) of 5 mM Gly-Ser at pD 7.4 and 60 °C.

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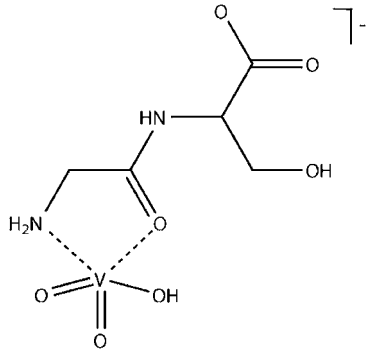
$$4V_1 \rightleftharpoons V_4$$

$$5V_1 \rightleftharpoons V_5$$

$$2V_1 \rightleftharpoons V_2$$

In order get more insight into the nature of the hydrolytically active species, the interaction between Gly-Ser and different oxovanadates was followed by ^{1}H, ^{13}C, and ^{51}V NMR spectroscopy. ^{51}V NMR spectra of vanadate solutions were recorded in the absence and the presence of 5.0 mM of Gly-Ser, and the data shown in Figure 3 indicate the appearance of a broad ^{51}V resonance at −506 pm, which is at the same position as previously reported for a complex formed between V_1 and Gly-Ser.[19] In this complex, designated Complex 1, the coordination of the peptide occurs via its amine nitrogen, amide nitrogen, and carboxylate oxygen. A detailed analysis of the ^{51}V NMR spectra indicated that while the V_2, V_4, and V_5 resonances remained unaffected upon addition of Gly-Ser, the V_1 resonance at −557.39 ppm is shifted by 0.57 ppm and broadened by 25 Hz at its half-width, suggesting its interaction with the peptide. The broadening and shifting of the V_1 resonance implies that another complex (designated Complex 2), that is in a fast exchange with the free Gly-Ser on the NMR time scale, is simultaneously formed. Further information about the structure of Complex 1 and Complex 2 was obtained by ^{1}H and ^{13}C NMR spectroscopy. Similarly to the ^{51}V NMR results, an additional set of Gly-Ser peaks was observed in both ^{1}H and ^{13}C NMR spectra, indicating formation of Complex 1. The NMR data shown in Table S3 are consistent with the coordination of Gly-Ser to vanadate via its amine nitrogen, amide nitrogen, and carboxylate oxygen as reported in previous studies, thus resulting in the structure of Complex 1 as shown in Scheme 5. The formation of Complex 1 was impeded with the protection of either the N-terminal or C-terminal of Gly-Ser, strongly suggesting that both the amine and carboxylate group are involved in complex formation. Interestingly, besides the appearance of a new set of peaks in ^{1}H and ^{13}C NMR, shifting of the Gly-Ser resonances was observed in both NMR methods. According to Table S4 the largest shifts were observed for the CH_2 (C1) and carbonyl C═O group (C2) of the glycyl residue, while the CH (C1′), CH_2 (C3′), and carboxylate COO^- group (C2′) of the serine residue were not affected by the addition of Na_3VO_4. The shift of the carbon C1 and C2 resonance suggests that Gly-Ser binds to vanadate via the amine nitrogen and the amide oxygen forming a complex designated Complex 2, which is in fast exchange with the free peptide on the NMR time scale (Scheme 4).

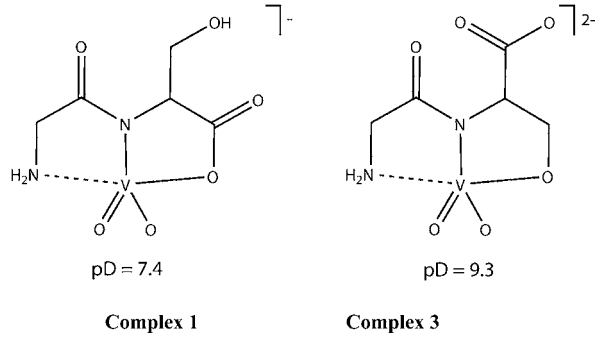
Scheme 4. Proposed Structure of Complex 2



Interestingly, hydrolysis of Ac-Gly-Ser in which the terminal amino function is converted into an amide was more than 10 times slower than the hydrolysis of Gly-Ser measured under the same reaction conditions (k_{obs} 1.04×10^{-7} s^{-1} vs 1.33×10^{-6} s^{-1}), which suggests that the coordination via amino group is essential for the efficient hydrolysis of the peptide bond. This in turn would suggest that in oligopeptides the cleavage would occur at the N-terminal amide bond.

Previous studies have shown that while at neutral pH the Gly-Ser binds to vanadate as shown in Complex 1, the increase of pH results in the formation of another complex, designated Complex 3, in which coordination of Gly-Ser occurs via its amine nitrogen, amide nitrogen, and hydroxyl oxygen. ^{51}V NMR spectra of Gly-Ser measured in the presence of vanadate at pD 9.3 indeed indicated the appearance of a new peak at −493 ppm, as reported in the literature.[17,19] ^{1}H and ^{13}C NMR studies (Table S5) supported the structure of Complex 3 shown in Scheme 5. The C3′ carbon experienced a large shift of 13.5 ppm, suggesting the coordination of the hydroxyl oxygen in the Ser side chain to vanadium.

Scheme 5. Proposed Structures of Complex 1 and Complex 3



Moreover, substitution of the amide nitrogen as in glycylsarcosine did not result in formation of Complex 2 and Complex 3, strongly suggesting the involvement of amide nitrogen in complex formation.[20] As previous studies suggested, these complexes have both a deprotonated amine (NH_2) nitrogen and a deprotonated amide (OCN^-) nitrogen.[21] Solid-state and solution studies confirm the loss of the amide proton in various other metal complexes.[36,37] With all the data above, the structures of two Gly-Ser complexes that are formed at different pH regimes were postulated as shown in Scheme 5.

Binding Vanadate to Gly-Ser. The increase of the chemical shift difference between the free and bound CH_2 group of the glycyl residue in Gly-Ser upon addition of Na_3VO_4 suggests the formation of a complex in which the dipeptide is in fast exchange between the free and bound form. The binding constant between vanadate and Gly-Ser in Complex 2 was determined by NMR titration experiments in which a fixed amount of Gly-Ser and increasing amounts of vanadate at pD 7.4 were used. The binding constant between Gly-Ser and vanadate was determined by fitting the data shown in Figure 4 to the scheme presented in eq 2.

The values for the Δ_{max} = 32 Hz, where Δ_{max} represents the maximum difference in ^{1}H NMR chemical shift between the free and bound CH_2 group of the glycyl residue in Gly-Ser occurring upon addition of Na_3VO_4 ($\Delta_{max} = \Delta_{boundGly\text{-}Ser} - \Delta_{freeGly\text{-}Ser}$) and the formation constant for Complex 2 [Gly-Ser/VO_4^{3-}] (K_f = 16.1 M^{-1}), were obtained by a computer

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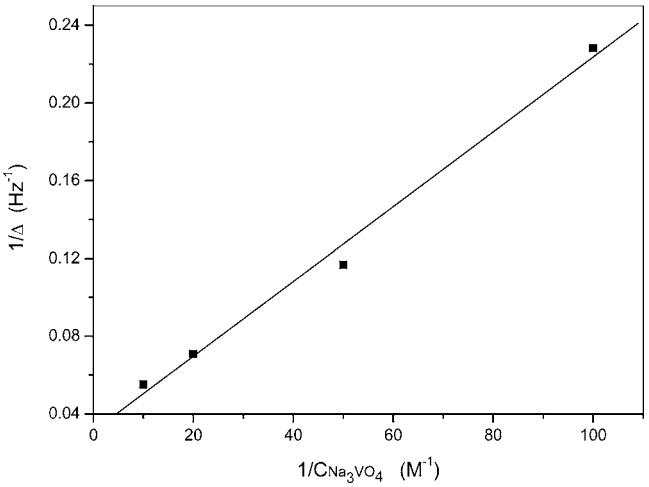

Figure 4. Effect of Na_3VO_4 concentration on the chemical shift difference between the free and bound CH_2 group of the glycyl residue in Gly-Ser.

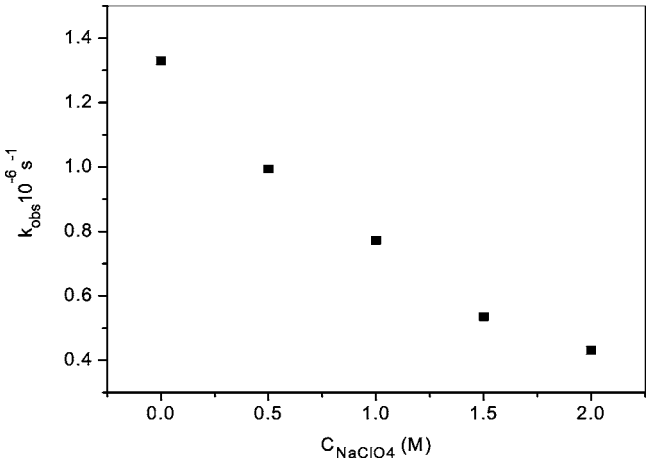

Figure 5. Influence of salt concentration on k_{obs} for the reaction between 2.0 mM Gly-Ser and Na_3VO_4 at pD 7.4 and 60 °C.

generated least-squares fit of k_{obs} to eq 3 which is applied for the binding in fast exchange[38]

$$\text{Gly-Ser} \overset{+[VO_4^{3-}]}{\rightleftharpoons} [\text{Gly-Ser...}VO_4^{3-}] \quad (2)$$

$$\frac{1}{\Delta} = \frac{1}{\Delta_{max} K_f [Na_3VO_4]} + \frac{1}{\Delta_{max}} \quad (3)$$

It is interesting to note that the value of the K_f is very similar to the formation constant previously determined for the binding between Gly-Gly and vanadate (17 M^{-1}).[17]

Since vanadate is known to interact with amino acids,[39] the interaction of vanadate with glycine and serine, which are the products of Gly-Ser hydrolysis, was also examined. ^1H NMR spectra of glycine and serine exhibited slight shifts upon addition of vanadate, indicating that weak interactions took place in solution. The binding of glycine and serine most likely occurs via their amine nitrogen and carboxylate oxygen which was confirmed by 0.01 ppm shifts of protons H_α of glycine and serine in the presence of vanadate (Table S6).

Effect of Reaction Conditions and Inhibitors on the Gly-Ser Hydrolysis. The influence of ionic strength on the rate constant was studied by following the hydrolytic reaction in the presence of different amounts of $NaClO_4$. A strong negative effect on the rate of Gly-Ser hydrolysis was observed upon adding increasing amounts of $NaClO_4$ (Figure 5). In the presence of 2.0 mM $NaClO_4$, the rate constant of the hydrolysis is ca. three times lower compared to the rate constant determined in the absence of $NaClO_4$. This may be due to the fact that the presence of high concentrations of salt impedes electrostatic interactions between vanadate and the peptide. However, addition of salt also influences the equilibrium between different oxovanadate species as it is known that in the presence of high concentrations of $NaClO_4$, polyoxovanadates V_4, V_5 forms become more prominent. Since ^{51}V NMR spectra indicated that vanadate is the species which effectively interacts with Gly-Ser, the decrease of its concentration in favor of polyoxo species may also influence the reaction rate.

The inhibitory effect of several nonreactive substrate analogues was examined by determining the rate constant for the hydrolysis of Gly-Ser (2.0 mM) by vanadate (20.0 mM) in the presence of an excess of several organic acids that are expected to bind to vanadate (Table 2). Among all the examined substrates only malonic, malic, and citric acid

Table 2. Rate Constants of Gly-Ser (2.0 mM) Peptide Bond Hydrolysis Measured in the Presence of Na_3VO_4 (20.0 mM) and Different Inhibitors (50.0 mM) at pD 7.4 and 60 °C

entry	inhibitor	formula	k_{obs}
1	no hibitor		1.33×10^{-6}
2	alanine	H_2NCH_2COOH	9.03×10^{-7}
3	malonic acid	$HOOCCH_2COOH$	7.22×10^{-7}
4	succinic acid	$HOOC(CH_2)_2COOH$	8.36×10^{-7}
5	glutaric acid	$HOOC(CH_2)_3COOH$	8.17×10^{-7}
6	adipic acid	$HOOC(CH_2)_4COOH$	9.22×10^{-7}
7	malic acid	$HOOCCH_2CH(OH)COOH$	7.53×10^{-7}
8	citric acid	$HOOCCH_2C(COOH)OHCH_2COOH$	5.47×10^{-7}

efficiently reduced vanadate promoted Gly-Ser hydrolysis. However, the binding of malonic and malic acid to vanadate appears to be rather weak, since a 25 molar excess of the inhibitor resulted only in respectively a 46 and 44% decrease of the rate constant. Due to the presence of an additional carboxylic group, the binding of citric acid to vanadate is stronger compared to malonic and malic acid, and, consequently, its presence resulted in a decrease of Gly-Ser hydrolysis by 58%. The inhibitory effect of other aliphatic dicarboxylic acids was rather small. Their bidentate mode of binding to vanadate is less effective as the tridentate binding of Gly-Ser that occurs via its amine nitrogen, amide oxygen, and carboxylate oxygen. These results are in accordance with the previous studies which suggest that binding of peptides to vanadate is 1−2 orders of magnitude stronger compared to the binding of oxalate and lactate.[19,40]

The effect of temperature on the hydrolytic reaction was determined by measuring the temperature dependence of k_{obs}. As expected, at higher temperatures increased rates were observed. The activation Gibbs function ($\Delta G^{\ddagger}$ = 117 kJ mol^{-1} at 37 °C), enthalpy ($\Delta H^{\ddagger}$ = 91 kJ mol^{-1}), and entropy ($\Delta S^{\ddagger}$ = −84 J mol^{-1} K^{-1}) were obtained from the Arrhenius and Eyring plots (Figure S3). The negative value of the activation entropy is in agreement with the formation of the complex [Gly-Ser/VO_4^{3-}]. However, the activation parameters need to be interpreted with caution, as they are usually derived from the

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composite rate constants that also include contribution from the binding of the substrate to the catalyst.

Mechanism of the Hydrolysis. ^{51}V NMR studies indicated that in a mixture containing different oxovanadate species, monomeric vanadate is the only anion that interacts with dipeptides. ^{1}H and ^{13}C NMR experiments indicated that monomeric vanadate can form three type of complexes with Gly-Ser, depending on the pH of the solution. While two complexes (Complex 1 and Complex 3) have been previously described, the existence of Complex 2 has not been reported so far. In Complexes 1 and 3 the amide nitrogen is coordinated to vanadate which leads to stabilization of the peptide bond.[17] This makes Complexes 1 and 3 very unlikely candidates to act as the hydrolytically active species. In contrary, in Complex 2, this coordination is not observed, and binding via the carbonyl oxygen is proposed. The interaction between the carbonyl oxygen of the peptide bond and metal ions is known to polarize this bond making the amide carbon more susceptible to nucleophilic attack of water which leads to peptide bond hydrolysis. The kinetic studies performed at different pD values suggest that although high pD should be beneficial for the presence of monomeric vanadate as it decreases the amount of inactive oligomeric forms, the reaction rate decreases presumably due to the formation of the inactive Complex 3.

From the screening experiments performed with a range of different dipeptides it was evident that the presence of the hydroxide group in the side chain of X-Ser type dipeptides was essential for efficient hydrolysis. As shown in Table S1 all dipeptides that were readily hydrolyzed have a serine residue at the C-terminus, suggesting the important role of the hydroxyl group in the Ser side chain as an intramolecular nucleophile. The hypothesis that the hydrolysis of X-Ser dipeptides is due to a N→O acyl rearrangement is strongly supported by the fact that hydrolysis of Gly-Ser was about 2 orders of magnitude faster than the hydrolysis of Ser-Gly (Table 3). While the

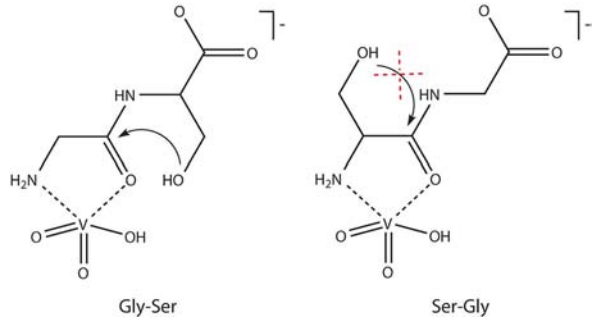
Table 3. Rate Constants of the Hydrolysis of Dipeptides in the Presence of Na_3VO_4 at pD 7.4 and 60 °C

peptide	k_{obs} (s^{-1})
Gly-Ser	1.33×10^{-6}
Leu-Ser	9.72×10^{-7}
His-Ser	1.15×10^{-6}
Gly-Ala	6.46×10^{-8}
Ser-Gly	5.22×10^{-8}
Gly-Ser-Phe	2.60×10^{-7}

intramolecular attack of the Ser hydroxyl group on the amide carbonyl carbon is possible in Gly-Ser, it is impossible in Ser-Gly. In the case of Gly-Ser the intramolecular attack of the Ser hydroxyl group results in a five-membered ring transition state, while in the case of Ser-Gly the intramolecular attack forms an unfavorable four-membered ring transition state (Scheme 6). In comparison to Gly-Ser, the hydrolysis of Gly-Ala in the presence of vanadate was approximately two order magnitude slower. The similarity in the backbone of Gly-Ser and Gly-Ala except for the hydroxyl group in the Ser side chain strongly suggests that this group is essential for effective hydrolysis.

The slower hydrolysis for Leu-Ser can be explained by the steric hindrance caused by the Leu side chain which inhibits nucleophilic attack and results in slower peptide hydrolysis. Similarly, in the case of Gly-Ser-Phe the presence of the bulky phenyl group in the side chain of the GlySerPhe tripeptide

Scheme 6. Intramolecular Attack of the Ser Hydroxyl Group on the Amide Carbonyl Carbon in Gly-Ser and Ser-Gly



sterically impedes the intramolecular attack of the Ser hydroxyl group on the amide carbonyl carbon.

The acceleration of Gly-Ser hydrolysis in the presence of vanadate suggested that this oxyanion, in addition to the hydroxyl group within the peptide, plays an important role in the hydrolysis of the dipeptide. The role of vanadate is most likely to polarize the carbonyl group by coordinating to it and to facilitate the attack of the nucleophile. All the data above allow to postulate a possible mechanism for the hydrolysis of Gly-Ser in the presence of vanadate as shown in Scheme 7.

A similar mechanism to that shown in Scheme 7 has been previously proposed for Zn(II)[25] and molybdate[34] assisted hydrolysis of GlySer. While the exact comparison is difficult to make due to the different conditions (pH, temperature, concentration, type of serine peptides) the rate of peptide hydrolysis in serine-containing peptides promoted by vanadate is in the same order of magnitude as previously reported for Zn(II) and molybdate salts.

Vanadate and Zn(II) have rather distinct coordination chemistry, however, they are both oxophilic[41] and have the tendency to coordinate to the carbonyl group and cause its polarization. Comparison of the Gly-Ser hydrolysis observed in the presence of molybdate and vanadate reveals that the rate constant measured in the presence of vanadate is about four times slower than that measured in the presence of molybdate under similar conditions.[34] While in the hydrolytically active Complex 2 formed by vanadate, only the bidentate coordination of Gly-Ser has been proposed, the tridentate coordination of the peptide has been observed in the hydrolytically active complex formed in the presence of molybdate. The different mode of coordination might be the cause for slightly higher hydrolytic activity of molybdate.

The gradual decrease in hydrolysis rate constant at pD > 7.4 can be explained by the increased formation of the hydrolytically inactive Complex 3, which starts to form around this pD. This complex has two unfavorable features for the hydrolysis of the peptide bond, as it involves coordination of the deprotonated amide nitrogen and hydroxyl oxygen to the metal ion. Substitution of the amide hydrogen atom for a metal greatly reduces the susceptibility of the amide carbonyl carbon atom toward nucleophilic attack, and, at the same time, coordination of the hydroxyl oxygen to the metal makes the nucleophilic attack to the amide bond impossible.

Our recent studies on the reactivity of oxovanadates toward carboxylic esters also identified monomeric vanadate as the hydrolytically active species.[14] The origin of the hydrolytic activity of vanadate was attributed to a combination of its nucleophilic nature and the chelating effect of the vanadium

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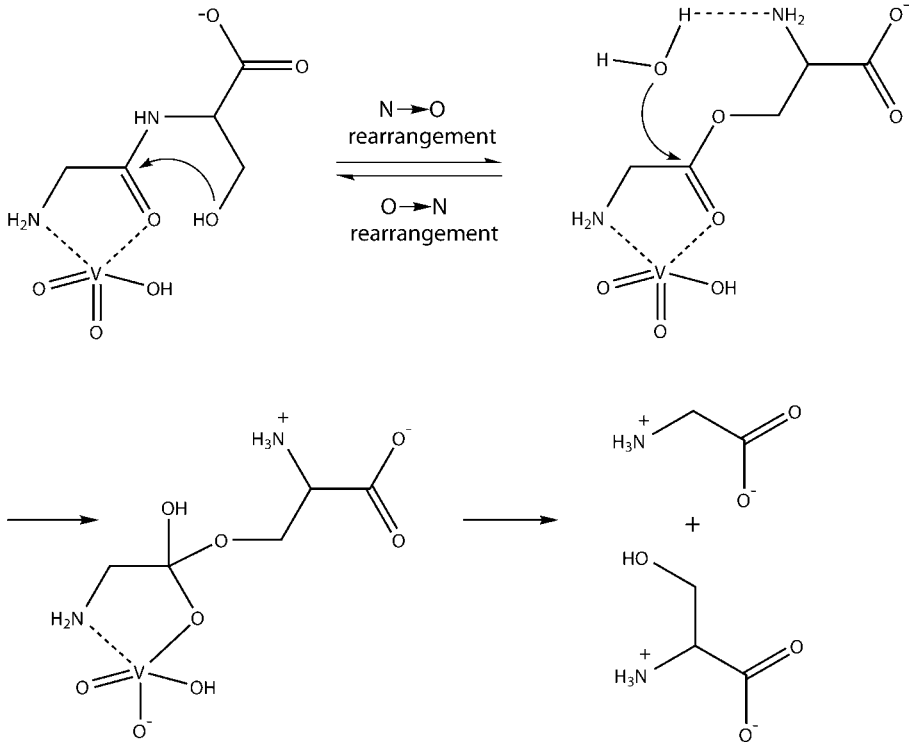
Scheme 7. Proposed Mechanism of Gly-Ser Hydrolysis Promoted by Vanadate



which can lead to the stabilization of the transition state. On the contrary, polyoxo forms of vanadate (V_4, V_5, and V_{10}) were implicated as active species in the hydrolysis of phosphoesters.[12,13] The high lability of the polyoxo forms results in partial detachment of one VO_4 tetrahedron and allows for the attachment of the structurally related phosphodiester tetrahedron into the polyoxometalate structure. The incorporation of the phosphodiester group into the polyoxovanadate skeleton results in the sharing of its oxygen atoms with V(V) center that can cause polarization and strain of the P−O ester bond, that in turn makes it more susceptible toward hydrolysis. The structural analogy between phosphate and vanadate forms the basis of this interaction; however, such interaction is less plausible between structurally distinct vanadate and peptides. Indeed, to the best of our knowledge there are no studies reporting on inclusion of peptides into polyoxovanadate structures. A complex formed between V_{10} and Gly-Gly has been structurally characterized, and it was found that the peptide interact noncovalently with the polyoxometalate structure via hydrogen bonding.[42] On the other hand, monomeric vanadate has a tendency to coordinate to polydentate ligands and to peptides.[17,39] Therefore, the different coordination affinities of monomeric and polyoxo forms of vanadate toward phosphoesters and peptides are most likely the reason for the different reactivity exhibited toward these classes of compounds.

Molecular Modeling of Gly-Ser and Its Vanadate Complexes. To support the experimental data and the suggestion that bidentate coordination of Gly-Ser to the vanadate ion through both the amine and carbonyl group might take place in the course of the hydrolysis reaction, we undertook DFT-based molecular modeling of Complex 2. For the sake of comparison the molecular structures of Complexes 1 and 3 as well as of that of the Gly-Ser anion and Gly-Ser zwitterion are also considered. Their lowest energy conformations optimized in aqueous media are depicted in Figure 6.

The theoretically predicted structures of Complexes 1 and 3 in the gas phase were previously described.[22] DFT-based Car−Parrinello molecular dynamics simulations and NMR calculations of the vanadate-glycylglycine complex that is analogous to Complex 1 reveal that in solution this compound should be formulated as five-coordinate anionic $[VO_2(Gly\text{-}Gly')]^-$

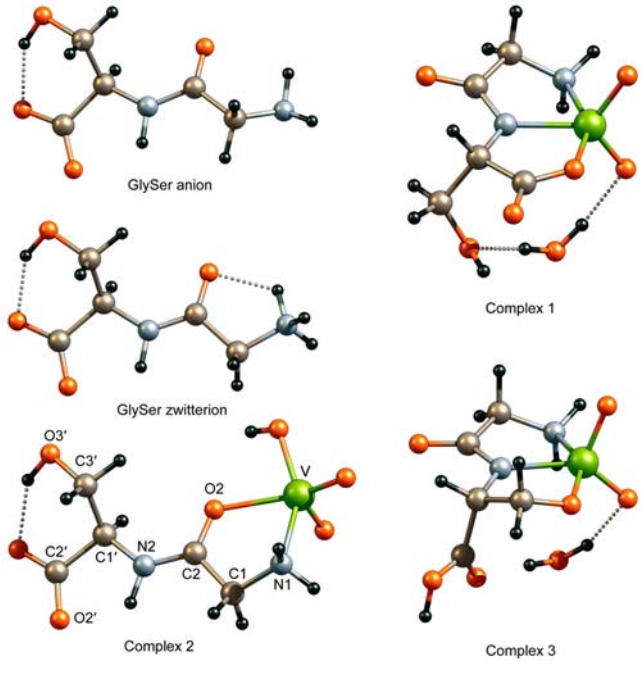


Figure 6. IEFPCM-B3LYP/6-311++G(2d,2p) optimized geometries of Gly-Ser and its vanadate complexes.

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Table 4. Experimental and Theoretical ^{1}H and ^{13}C NMR Chemical Shifts (δ, ppm) Calculated at the IEFPCM-B3LYP/6-311++G(2d,2p) Level of Theory

	Gly-Ser anion		Gly-Ser zwitterion		complex 2	complex 1		complex 3	
atom	exp.	theor.	exp.	theor.	theor.	exp.	theor.	exp.	theor.
C1	42.97	49.81	40.76	45.16	49.57	46.84	52.37	47.90	53.83
C2	172.61	179.37	167.10	169.76	183.87	179.80	186.53	172.56	186.90
C1′	57.02	58.20	57.29	59.58	59.53	67.94	78.03	69.83	74.63
C2′	175.91	184.54	175.97	182.84	182.27	182.99	189.69	179.59	187.71
C3′	62.11	68.18	62.0	67.75	67.35	62.26	71.58	75.60	84.05
Ha (average)	3.49	3.39/3.72 (3.55)	3.85	3.92/3.94 (3.93)	3.68/3.84 (3.76)	3.59	3.51/3.53 (3.52)	3.60	3.43/3.55 (3.49)
Ha′	4.29	4.06	4.33	4.09	4.14	4.69	5.04	4.58	5.14
Hb′ (average)	3.80/3.84	3.35/3.98 (3.67)	3.82/3.88	3.49/4.08 (3.79)	3.52/4.12 (3.82)	3.95/4.11	4.20/4.41 (4.31)	4.39/4.55	4.48/5.32 (4.90)

Table 5. Relative Experimental and Theoretical ^{1}H and ^{13}C NMR Chemical Shifts ($\Delta\delta$, ppm) Calculated at the IEFPCM-B3LYP/6-311++G(2d,2p) Level of Theory

	complex 2		complex 1		complex 3	
atom	exp.	theor.[a]	exp.	theor.[a]	exp.	theor.[b]
C1	0.16	4.41	6.08	7.21	4.93	4.03
C2	0.37	14.11	12.70	16.77	−0.05	7.53
C1′	−0.01	−0.06	10.65	18.45	12.81	16.42
C2′	0.03	−0.57	7.02	6.85	3.68	3.17
C3′	0.02	−0.40	0.26	3.83	13.49	15.87
Ha	0.02	−0.24/−0.10	−0.26	−0.40/−0.41	0.11	0.05/−0.17
Ha′	0	0.05	0.36	0.95	0.29	1.07
Hb′	0	0.03/0.04	0.13/0.23	0.71/0.32	0.59/0.71	1.13/1.35

[a]Calculated with respect to Gly-Ser zwitterion. [b]Calculated with respect to Gly-Ser anion.

species.[23] Based on these findings in this study Complexes 1−3 are modeled as anionic species with a five-coordinated vanadium. Among the three complexes considered Complex 1 has the lowest free energy in the aqueous phase. As it was previously found[39] the tridentate coordination of Gly-Ser through N1, N2, and O3 in complex 3 is less stable than the corresponding N1, N2, and O2 coordination in Complex 1. The free energy in solution of Complex 3 is calculated to be higher with respect to complex 1 by 8.4 kcal/mol. Noteworthy, the free energy of Complex 2 is computed to be higher with respect to the major complex (Complex 1) by only 7.1 kcal/mol. Thus, on the basis of the calculations the formation of Complex 2 should be thermodynamically feasible under the reaction conditions.

Further, in support of the experimental evidence for bidentate coordination of Gly-Ser the ^{1}H and ^{13}C NMR chemical shifts for the three complexes and the free ligand were calculated and compared with the experimental spectra. The theoretical chemical shifts along with the experimental data are summarized in Table 4. A comparison of the results for the Gly-Ser anion (pD = 9.3) and Gly-Ser zwitterion (pD = 7.4) as well as for the Complexes 1 and 3 reveal very good agreement between the calculated and the experimental ^{1}H and ^{13}C NMR shifts. The ^{13}C NMR chemical shifts for these species are predicted with a standard error of 2−3 ppm and a R^2 = 0.99. However, a better understanding of the NMR changes in Gly-Ser which occur upon coordination could be obtained considering the relative chemical shifts, $\Delta\delta$ (Table 5). With exception of $\Delta\delta$ values for C1 in complex 2 and for C2 in complex 3 all other theoretical values are in qualitative agreement with the experiment. It should be noted that for Complex 2 the calculations predict the largest $\Delta\delta$ value for C2 followed by C1 and almost no shifting for the other atoms. Qualitatively this is exactly what is experimentally observed, however in smaller magnitude. A possible explanation for the smaller experimental shifting of C2 and C1 could be found on the basis of lower kinetic stability of Complex 2 and comparatively short lifetime of this complex.

For a detailed understanding of the role of the vanadate or how Gly-Ser becomes hydrolytically active/inactive upon coordination a comparative analysis of various molecular properties of Gly-Ser and its vanadate complexes were performed. Selected geometrical parameters, atomic charges, bond orders, and the bonding orbitals polarization (from NBO analysis) are collected in Table 6. A comparison of the bonding orbitals polarizations (BOP) of Gly-Ser with those of Complexes 1−3 reveals that the σC2−N2 and σC2−O2 bonding orbitals remain slightly affected upon coordination, whereas the πC2−O2 is more sensitive and hence more informative. The coordination of the Gly-Ser carbonyl oxygen to the vanadium atom produce additional polarization of the C═O bond. Thus, the πC═O bonding orbital, with percentage of occupancy 26.18(C)/73.82(O) in the Gly-Ser zwitterion, becomes more polarized in Complex 2, 22.29(C)/77.71(O), the C═O bond order decreases from 1.55 to 1.40, and the bond length increases with 0.021 Å. Conversely, going again from the Gly-Ser zwitterion to Complex 2, the C2−N2 bond order increase from 1.31 to 1.37 and the C2−N2 bond length is calculated to be shorter by 0.011 Å. As a result of electron density reorganization upon coordination the atomic charge of C2 in Complex 2 becomes more positive, as compared to the free ligand, and hence more susceptible for nucleophilic attack (in particular, intramolecular attack of the alcoholic function). In Complexes 1 and 3 the atomic charges

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Table 6. Relative Free Energy in Aqueous Solution (ΔG_{sol}, kcal/mol), Selected Geometrical Parameters (Bond Lengths in Å, Angles in deg), Wiberg Bond Order, Bonding Orbitals Polarization (BOP, %), and Atomic Charges Calculated at the IEFPCM-B3LYP/6-311++(2d,2p) Level of Theory

quantity	Gly-Ser anion	Gly-Ser zwitterion	complex 2	complex 1	complex 3
ΔG_{sol}			7.1	0.0	8.4
Geom. param.					
R(C2−N2)	1.345	1.328	1.317	1.341	1.337
R(C2−O2)	1.234	1.235	1.256	1.238	1.241
R(N3−C1′)	1.452	1.456	1.458	1.454	1.445
R(O2. O2′. O3′)−V	-	-	2.153	1.961	1.886
R(N1−V)	-	-	2.212	2.155	2.182
R(N2−V)	-	-	-	2.047	2.072
D(O2−C2−N2−C1′)	4.3	6.3	6.5	−2.2	0.3
Wiberg BO					
C2−N2	1.25	1.31	1.37	1.29	1.30
C2−O2	1.57	1.55	1.40	1.54	1.53
(O2. O2′. O3′)−V	-	-	0.38	0.63	0.83
N1−V	-	-	0.43	0.51	0.48
N2−V	-	-	-	0.62	0.59
NBO BOP					
C2−N2	σ 37.64/62.36	σ 38.26/61.74	σ 38.32/61.68	σ 38.34/61.66	σ 38.38/61.62
C2−O2	σ 34.34/65.66	σ 35.31/64.69	σ 34.46/65.54	σ 34.92/65.08	σ 34.92/65.08
	π 27.43/72.57	π 26.18/73.82	π 22.29/77.71	π 26.31/73.69	π 26.03/73.97
Charge					
NBO					
C2	0.691	0.683	0.715	0.684	0.684
N2	−0.627	−0.604	−0.572	−0.641	−0.638
O2	−0.721	−0.699	−0.638	−0.730	−0.740
V	-	-	0.895	0.849	0.837
Hirshfeld					
C2	0.166	0.185	0.214	0.156	0.148
N2	−0.079	−0.062	−0.047	−0.180	−0.176
O2	−0.359	−0.307	−0.231	−0.384	−0.403
V	-	-	0.465	0.441	0.401

of C2 retain almost the same values (according to NBO analysis) or become even less positive (according to Hirshfeld analysis) as compared to the free ligand.

CONCLUSION

In conclusion, we report on the first example of peptide bond hydrolysis promoted by the vanadate(V) oxyanion. The hydrolytic effect of vanadate can be attributed to its ability to efficiently coordinate X-Ser peptides and polarize carbonyl groups toward the internal nucleophilic attack by a hydroxyl group of Ser residue. Although the hydrolysis occurred in solutions containing polyoxo-forms of vanadate, the NMR and kinetic studies indentified the monomeric vanadate as the source for the hydrolytically active species. A previously unreported complex in which coordination of the peptide to vanadate occurs via amine nitrogen and peptide carbonyl oxygen atoms has been discovered. This complex is proposed to be the effective hydrolytic species, and its existence has been supported by theoretical calculations which suggest that the formation of Complex 2 is thermodynamically feasible under the reaction conditions. We are currently investigating the reactivity of vanadate toward the hydrolysis of a range of different oligopeptides and proteins. It is interesting to mention that although the studies with short peptides presented in this work suggest that the cleavage should preferentially occur at the N-terminal amide bond, the preliminary studies with proteins indicate that the hydrolysis of the internal amide bonds is also possible. While the aim of our studies is not necessarily to propose the use of vanadate for practical purposes in the hydrolysis of peptides and proteins, but it is a rather a remarkable fundamental finding that despite the lack of some basic requirements commonly assumed for an artificial metallopeptidase such as positive charge and the presence of water or hydroxide in the first coordination spehre, the vanadate anion is able to selectively hydrolyze a series of X-Ser peptides under physiological pH and temperature. This and future studies focusing on the reactivity of oxovanadates toward relevant biomolecules may shed more light on the molecular origin of the biological activity of vanadium.

EXPERIMENTAL SECTION

Materials. Glycylserine (Gly-Ser), leucylserine (Leu-Ser), histidylserine (His-Ser), glycylalanine (Gly-Ala), serylglycine (Ser-Gly), and sodium vanadate were purchased from Acros and used without further purification. The pH of the solutions for the ^{1}H NMR studies was adjusted with D_2SO_4 and NaOD, both from Acros.

NMR Spectroscopy. ^{1}H, ^{13}C, and ^{51}V NMR spectra were recorded on a Bruker Advance 400 and Bruker Advance 600 spectrometers. D_2O with 0.05 wt % 3-(trimethylsilyl)propionic acid as an internal standard for ^{1}H NMR was used as a solvent. A solution of $VOCl_3$ and a solution of TMS in $CDCl_3$ were employed as an external ^{51}V and ^{13}C NMR references.

Kinetics. In a typical kinetic experiment, the hydrolysis of 2 mM a peptide in the presence of 25 mM of vanadate was followed by ^{1}H NMR spectroscopy. For example, the typical reaction mixture was prepared by dissolving 0.32 mg of Gly-Ser in 1 mL of D_2O to which

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4.6 mg of $Na_3VO_4 \times 2\ H_2O$ was added. The pH of the final mixture was adjusted by adding small amounts (typically 1−5 μL) of 2 M D_2SO_4 or 1.5 M NaOD. The pH of the solution was measured in the beginning and at the end of the hydrolytic reaction, and the difference was typically less than 0.1 unit. The pD value of the solution was obtained by adding 0.41 to the pH reading, according to formula pD = pH + 0.41. The reaction samples were kept at constant temperature (typically 60 °C), and the rate constants for the hydrolysis were determined by following the appearance of the free glycine resonance in the ^{1}H NMR spectra at different time intervals. The observed first order rate constants (k_{obs}) were calculated by the integral method from at least 90% conversion. This included integrating proton NMR resonance of free glycine and plotting them as a function of time. The linear fitting method ($\ln[A] = k_{obs} \times t + C$), where A is the concentration of the substrate and t is the time at which concentration was measured, was used. The R-values were generally higher than 0.98. The influence of the ionic strength on the reaction rate was studied by following the reaction between 2 mM Gly-Ser and 25 mM Na_3VO_4 (pD 7.0, 60 °C) in the presence of different amounts of $NaClO_4$. As before, the rate constants were determined by the integration of the ^{1}H NMR signal intensities of the CH_2 group of free glycine.

Computational Procedure. All geometrical optimizations and molecular properties were calculated with the Gaussian09[43] program package. Conformational analysis of the Gly-Ser anion, Gly-Ser zwitterion, and Complexes 1, 2, and 3 were performed with the hybrid density functional B3LYP[44−46] and basis 6-31+G(d,p) both in the gas phase and in water solution, utilizing the IEFPCM[47,48] method as implemented in Gaussian09 (using the default options). The vanadium electrons in Complexes 1−3 were described with relativistic Stuttgart pseudopotential[49] (SDD, describing ten core electrons) and the appropriate contracted basis set (8s7p6d1f)/[6s5p3d1f]. The lowest energy conformations were refined with the same density functional in solution and basis set 6-311++G(2d,2p) for all atoms. The molecular properties were calculated at the final geometries using the same level of theory, IEFPCM-B3LYP/6-311++G(2d,2p). To estimate the atomic charges both Hirshfeld[50−52] and natural bond orbital (NBO) analysis[53,54] were performed. The polarization of the bonding orbitals and the Wiberg bond orders[55] are computed applying the NBO scheme. The ^{1}H and ^{13}C isotropic shielding constants (σ_i) were computed using the gauge-independent atomic orbital (GIAO) method.[56,57] They were used to obtain the chemical shifts ($\delta_i = \sigma_{TMS} - \sigma_i$) by referring to the standard compound tetramethylsilane (TMS).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Tables S1−S7 and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: Tatjana.Vogt@chem.kuleuven.be.

Notes

The authors declare no competing financial interest.

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