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Mononuclear and dinuclear peroxotungsten complexes with co-ordinated dipeptides as potent inhibitors of alkaline phosphatase activity

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Abstract

New molecular peroxotungstate(VI) complexes with dipeptides as ancillary ligands of the type, $[WO(O_2)_2(dipeptide)(H_2O)].3H_2O$, dipeptide = glycyl-glycine or glycyl-leucine, have been synthesized and characterized by elemental analysis, spectral and physico-chemical methods including thermal analysis. The complexes contain side-on bound peroxo groups and a peptide zwitterion bonded to the metal centre unidentately through an O(carboxylate) atom. Investigations on certain biologically important key properties of these compounds and a set of dimeric compounds in analogous co-ligand environment, $Na_2[W_2O_3(O_2)_4(dipeptide)_2].3H_2O$, dipeptide = glycyl-glycine and glycyl-leucine, reported previously by us revealed interesting features of the compounds. Each of the compounds despite having a 7 co-ordinated metal centre exerts a strong inhibitory effect on alkaline phosphatase activity with a potency higher than that of the free dipeptide, tungstate or peroxotungstate. The compounds exhibit remarkable stability in solutions of acidic as well as physiological pH and are weaker as substrate to the enzyme catalase, compared to H_2O_2. The mononuclear and dinuclear peroxotungsten compounds are efficient oxidants of reduced glutathione (GSH), a reaction in which only one of the peroxo groups of a diperoxotungsten moiety of the complexes was found to be active.

Keywords: *ALP inhibitor, peptide containing peroxotungstate, GSH oxidant, substrate to catalase, alkaline phosphatase, inhibition*

Introduction

Peroxotungsten complexes have been receiving continued importance mainly owing to their application as an important class of stoichiometric or catalytic oxidizing and oxo-transfer agent in a variety of organic oxidations [1–8]. There has been a revival of interest in peroxo-tungsten systems since it has been demonstrated that tungstates and peroxotungstates (pW) present in a solution of $W\text{-}H_2O_2$, like vanadate and peroxovanadates (pV), were capable of inhibiting the hydrolysis of phosphoproteins [9–13] and mimic the insulin bioeffects in rat adipocytes [10]. Moreover, with the exciting discovery of several tungstoenzymes in recent years biological relevance of tungsten and its compounds has finally been recognized [14,15]. Recent findings on the ability of the compounds of tungsten such as polyoxotungstates and silicotungstates to show antiviral activity [16,17] and to be potent inhibitors of HIV reverse transcriptase and RNA-dependant DNA polymerase [17] are exciting contributions to the current knowledge of biochemical importance of the metal and its compounds. The importance of enzyme inhibition as a mode of action for inorganic drugs is being realized in recent years and is an area needing exploration [16].

Although much research has been performed on the potential usage of compounds of vanadium as therapeutic antidiabetic agents [18–21], the detailed mechanism by which vanadates and peroxovanadates induce their insulin-mimetic effect or inhibit enzyme functions is yet to be fully understood [22–24].

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A definite correlation was found to exist between abilities of vanadate and pV to inhibit protein phosphatases and their in vivo insulin mimetic activities [22–24]. However, most of the synthetic pV compounds tested for their various biochemical effects suffer from disadvantage of being hydrolytically unstable and this limits their utility as therapeutic agents [18,19,24]. It is notable in this context that peroxotungstates, formed in a solution of W-H_2O_2 not only exhibited insuline-like behaviour but were also found to be stable in solution of a wide range of pH values [10]. Surprisingly, despite these important findings to the best of our knowledge there has been no information available involving the effect of well-defined synthetic heteroligand peroxotungsten compounds on activity of different enzymes including phosphatases [16], until we reported our observations on the phosphatase inhibitory effect of the dinuclear pW compounds, $A_2[W_2O_3(O_2)_4(\text{cystine})].4H_2O$ (A = Na or K), with a potency significantly higher than that of the free cystine, tungstate, or peroxotungstate [25]. Most importantly, these complexes with co-ordinated cystine, were found to be highly stable in solution of acidic as well as alkaline pH, served as a weak substrate to catalase, and efficiently oxidized GSH to GSSG [25].

As a direct sequel to this and in order to gain a better insight into the afore mentioned aspects of pW systems, we considered it imperative to generate further information regarding biologically relevant properties of new monomeric and dimeric pW compounds in biogenic co-ligand environment. Pertinent here is to mention that work on pW compounds containing biogenic species such as amino acids and peptides as ancillary ligands have so far received scant attention [4,26,27]. We have already gained an access to first peptide containing pW compounds of the type, $Na_2[W_2O_3(O_2)_4(\text{dipeptide})_2].3H_2O$, dipeptide = glycyl-glycine (**1**) and glycyl-leucine (**2**), which proved to be efficient oxidant of bromide at physiological pH [26], an essential requirement of biomimetic model. In the present study therefore, we have been specifically interested to obtain monomeric pW analogues of these compounds, which would also enable us to investigate whether monomeric and dimeric pW compounds in similar co-ligand environmrnt would exhibit similarities in their redox and solution properties.

In this paper, we report the synthesis and characterization of new molecular peroxo compounds of W(VI)), $[WO(O_2)_2(\text{dipeptide})(H_2O)].3H_2O$, dipeptide = glycyl-glycine (**3**) and glycyl-leucine (**4**). The thermal stability of the newly synthesized compounds as well as their stability towards decomposition in solution have been examined and compared with their dimeric analogues. We have investigated the redox activity of the mononuclear and dinuclear compounds **1**–**4** with respect to GSH and their interaction with catalase. Effects of the two types of pW complexes **1**–**4** upon alkaline phosphatase (ALP) activity have been determined.

Materials and methods

Chemicals and solutions

The sources of chemicals are given below: Catalase, alkaline phosphatase from rabbit intestine, *p*-nitrophenyl phosphate (p-NPP) and glycyl-peptides (Sigma-Aldrich Chemical Company, New Delhi); sodium and potassium tungstates, (CDH, New Delhi,India); hydrogen peroxide (30%) (Ranbaxy, New Delhi, India); dithionitrobenzoic acid (DTNB), glutathione (Himedia laboratories, Mumbai, India); glycine, potassium dihydrogen phosphates, sodium and potassium hydroxides, magnesium chloride (SD Fine Chemicals, Mumbai, India). The complexes $Na_2[W_2O_3(O_2)_4(\text{gly-gly})_2].3H_2O$ (**1**) and $Na_2[W_2O_3(O_2)_4(\text{gly-leu})_2].3H_2O$ (**2**) were prepared by methods reported earlier [26]. The water used for solution preparation was deionized and distilled.

*Synthesis of monomeric peroxotungstate complexes, $[WO(O_2)_2(\text{glycyl-glycine})(H_2O)].3H_2O$ (**3**) and $[WO(O_2)_2(\text{glycyl-leucine})(H_2O)].3H_2O$ (**4**):*

The procedure adopted for the synthesis is common to both complexes. This consisted of gradual addition of 5 mL H_2O_2 (30% solution, 44 mmol) to a mixture of H_2WO_4 (0.5 g, 1.5 mmol) and peptides at a molar ratio of W: dipeptide of 1:1 with continuous stirring. Keeping the temperature below 4°C in an ice bath, the mixture was stirred for ca. 5 min until all solids dissolved. At this stage the pH of the solution was recorded to be ca. 1.5. The pH of the reaction mixture was raised up to 5.5 by adding NaOH solution (0.1 M) dropwise. On adding pre-cooled acetone (about 50 mL) to the above solution under continuous stirring a colorless pasty mass separated out. After allowing to stand for about 10 min in the ice bath, the supernatant liquid was decanted off and the residue was treated repeatedly with distilled acetone under scratching until it became a white microcrystalline solid. The product was separated by centrifugation, washed with cold distilled acetone and dried in vacuo over concentrated sulfuric acid.

*Anal. Calc. for $[WO(O_2)_2(\text{gly-gly})(H_2O)].3H_2O$ (**3**):*. W, 39.40; O_2^{2-}, 13.70; C, 10.27; N, 5.99; H, 3.42. Found: W, 38.85; O_2^{2-}, 13.56; C, 10.57; N, 5.73; H, 3.12. Yield: approximately 45%

*Anal. Calc. for $[WO(O_2)_2(\text{gly-leu})(H_2O)].3H_2O$ (**4**):*. W, 35.11; O_2^{2-}, 12.21; C, 18.32; N, 5.34; H, 4.58. Found: W, 35.28; O_2^{2-}, 12.12; C, 18.25; N, 5.40; H, 3.86. Yield: approximately 48%

Elemental analysis. The compounds were analyzed for C, H, and N at the Regional Sophisticated Instrumentation Centre (RSIC), North Eastern Hill University, Shillong, India. The total peroxide content was determined by adding a weighed amount of the compound to a cold solution of 1.5% boric acid (W/V) in 0.7 M sulfuric acid (100 mL) and titration with standard Cerium (IV) solution [28]. Tungsten was determined gravimetrically [29] as $BaWO_4$. The values are given as % by weight of the compounds from which the ratios of metal: peroxide are derived.

Physical and spectroscopic measurements. The IR spectra were recorded with samples as KBr pellets in a Nicolet model 410 FTIR spectrophotometer. The spectra were recorded at ambient temperature by making pressed pellets of the compounds. Spectroscopic determinations of the initial rate of ALP catalyzed hydrolysis of p-NPP and oxidations of GSH were carried out in a Cary model Bio 100 spectrophotometer, equipped with a peltier controlled constant temperature cell. The absorbance values were denoted as e.g. A_{405} at the wavelength indicated. Thermogravimetric analysis was done in Mettler Toledo Star system at a heating rate of 5°C/min under the atmosphere of nitrogen using aluminum pan.

*Stability of complexes **3** and **4** in solution*. Stability of the compounds in distilled water at pH ca. 5, the natural pH of the solutions of the compounds in water, was studied by estimating the peroxide content in aliquots drawn from a solution of the compounds (0.1 mM) at different time intervals by the method described above (Figure 2). As a measure of stability of the compounds in solution change in absorbance of their electronic spectral bands at ambient temperature were recorded at 30 min gap for a period of 12 h. Stability of the compounds at pH 7.0 or 8.0 was measured similarly in compound solution (0.1 mM) in phosphate buffer (50 mM, pH 7.0 or 8.0). In order to measure the stability of the compounds at acidic pH citrate buffer was used (pH 3.4).

*Effect of catalase on the complexes **1**– **4***. The effect of catalase on complexes was studied by estimating the peroxide content of the compounds in a solution containing catalase at specified time intervals (Figure 2). The test solution contained phosphate buffer (50 mM, pH 7.0) and catalase (40 μg/mL). The volume of the reaction solution was kept at 25mL. The solution was incubated at 30°C. The compound was then added to the test solution and aliquots of 5 mL were pipetted out and titrated for peroxide content after stopping the reaction by adding it to cold sulfuric acid (0.7 M, 100 mL) at time 5, 10, 15, 20, 25 and 40 min of starting the reaction. Three concentrations of peroxo compounds (0.05, 0.1, 0.2 mM) were tested.

Measurement of redox activity in solution. To a reaction mixture containing GSH (80 μM) and phosphate buffer (50 mM, pH 7.0) a measured amount of aliquot from solution of the synthesized compound (1 mM) was added. Following incubation of 10 min, DTNB (160 μM) was added to the solution and the change in absorbance at 412 nm was determined in order to measure the GSH remaining in solution by the method of Ellman [30] using molar extinction of $\varepsilon_{412} = 13{,}600$. Measurements were done by using different concentrations of the compounds, 5.0 and 10.0 μM, in triplicate under same assay conditions.

Measurement of alkaline phosphatase activity. Phosphatase activity was assayed spectrophotometrically by using p-nitrophenyl phosphate (p-NPP) as a substrate. The continuous production of p-nitrophenol (p-NP) was determined at 30°C by measuring absorbance at 405 nm in a reaction mixture containing ALP from rabbit intestine (3.3 μg protein/mL), p-NPP (1 mM) in incubation buffer (25 mM glycine + 2 mM $MgCl_2$, pH 10.0). The initial reaction rates were obtained by starting the reaction by adding ALP to the reaction solution, which was pre-incubated for 5 min. The initial reaction rate of p-NPP hydrolysis in the absence of the inhibitors, V_0 was determined which was used as control. The effects of peroxotungstate and other inhibitors were assessed by adding different concentrations (10–100 μM) of each species in the ALP assay. The V_i was obtained as the rate of p-NPP hydrolysis in the presence of variable concentrations of inhibitors under similar experimental conditions. The V_0/V_i ratios were calculated from these values. The concentrations tested for the compounds and each of the other inhibitors were 10, 20, 30, 40, 50 and 75 μM. The IC_{50} values were graphically determined as the half-maximal inhibitory concentration of the inhibitor species giving 50% inhibition. All the assays were performed in triplicate. The data in figures are presented as the means ± SE from three separate experiments.

Results

Synthesis and characterization

The synthesis of monomeric peroxotungsten compounds **3** and **4** has been achieved from the reaction of H_2WO_4 with 30% H_2O_2 and the respective ligand at near neutral pH of ca. 5. The procedure included other essential components such as maintenance of required time and temperature at ≤ 4°C and limiting water to that contributed by 30% H_2O_2 and alkali hydroxide solution.

Table I. Infrared and ultraviolet spectral data of compounds **3** and **4**.

Compound	IR peak (cm^{-1})				UV peak (nm)
	ν(W=O)	ν(O-O)	ν_{as}(W-O_2)	ν_s(W-O_2)	
[WO(O_2)$_2$(gly-gly)(H_2O)].3H_2O (**3**)	957	875	615	539	254
[WO(O_2)$_2$(gly-leu)(H_2O)].3H_2O (**4**)	956	876	593	540	252

The complexes were obtained as white microcrystalline products, hygroscopic in nature. In the solid state they remained stable for several weeks when stored dry in closed containers at temperature $< 30°C$. The elemental analysis data for the compounds **3** and **4** indicated the presence of two peroxide groups and one peptide ligand per metal centre which could be fitted with the formulation of the complex species as [WO(O_2)$_2$(dipeptide)(H_2O)].3H_2O, dipeptide = glycyl-glycine (**3**) and glycyl-leucine (**4**).

The IR spectra of the compounds **3** and **4** gave clear indication of the presence of co-ordinated peroxide, co-ordinated peptide, terminally bonded W=O in each of them. The significant general features are presented in Table I. The occurrence of side-on bound peroxo ligand in these compounds was evident from the observance of the characteristic ν(O-O), ν_{as}(W-O_2) and ν_s(W-O_2) modes, in the vicinity of ca. 870, ca. 610 and ca. 530 cm^{-1}, respectively. The spectra enabled clear identification of ν(W=O) near 960 cm^{-1} arising from terminally bonded W=O group [31,32].

The IR spectra of glycyl-glycine and its compounds have been extensively studied in solution [33–36] as well as in solid state [33]. A clear resemblance was observed between the IR spectral pattern originating from co-ordinated peptides of the monomeric compounds **3**, **4** and the corresponding dinuclear peroxotungstates **1** and **2** [26]. The spectra of the complexes **3** and **4,** exhibited two distinct bands in the range of 1680 − 1660 cm^{-1} and 1630–1600 cm^{-1} which have been assigned to ν(C=O) (amide) and ν_{as}(COO) of the co-ordinated peptide ligands [33–36]. The position of ν(C=O) band remained almost unaltered compared to its position in free glycyl-glycine (1675 cm^{-1}) or glycyl-leucine (1690 cm^{-1}), which indicated that the amide group was not taking part in co-ordination. Bonding through N-atom of the amide group was unlikely as evident from the spectra because such co-ordination is known to cause considerable decrease in the peptide carbonyl stretching frequency, which was not observed in case of these compounds [37,38]. The ν_s(COO) vibration of the free ligands were observed in the range of 1410 cm^{-1} (glycyl-leucine) and 1395 cm^{-1} (glycyl-glycine) in the IR spectra [32]. A medium intensity band with some broadening observed at ca. 1380–1390 cm^{-1} region was assigned to ν_s(COO) of the unidentate carboxylate group ($\nu_{as} - \nu_s \approx 250$ cm^{-1}) [37]. The broadening of the band was probably caused by its mixing with the C–N stretching of amide group expected to occur in this region [36]. The spectra showed N-H stretching bands of coordinated peptide residue at 3300–3100 cm^{-1} regions as expected from the -N^+H_3 group. The rocking modes of -N^+H_3 occurred at ca.1130 and ca.1042 cm^{-1}. The presence of water in the complexes was evident from the broad absorption at 3500–3400 cm^{-1}, due to ν(OH). Owing to the presence of lattice water, IR spectral information on ν(OH) and δ(H-O-H) modes are not very significant in so far as the distinction between co-ordinated and lattice water are concerned. Fortunately, a consistent appearance of a medium intensity signal at ca. 725 cm^{-1} attributable to rocking mode of water indicated the presence of co-ordinated water in each of the compounds. The IR spectral data thus suggest that in the complexes **3** and **4**, dipeptide ligand occurring as zwitterion binds the metal center through O(carboxylate) atom. A co-ordinated water molecule completes hepta co-ordination in each case. Based on these data a structure of the type shown schematically in Figure 1 has been envisaged for complexes, **3** and **4** which is shown for the glycyl–glycine complex as a representative example.

Electronic spectra of compounds **3** and **4** in aqueous solution exhibited a weak intensity broad band at 240–250 nm (Table I) originating from co-ordinated peroxide. These bands are typical of LMCT transitions of diperoxo derivative of tungsten [4,8].

Thermal analysis

Thermogravimetric analysis data indicated that after the initial dehydration, the compounds **3** and **4**

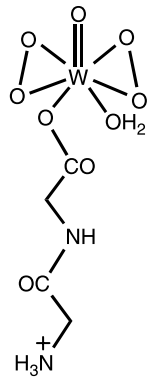

Figure 1. Proposed structure of the monomeric peroxotungstate compounds shown with [WO(O_2)$_2$(gly-gly)(H_2O)].3H_2O **(3)** as representative.

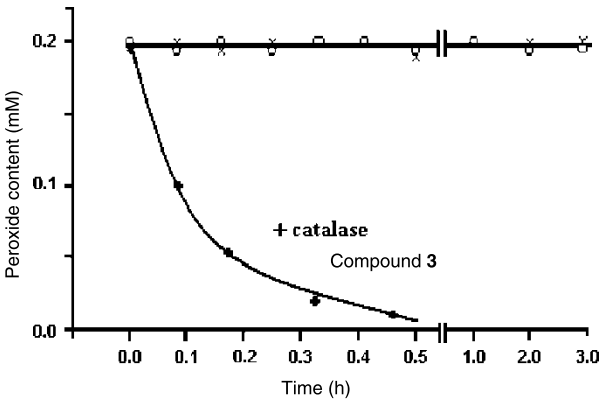

Figure 2. Stability of complexes at different pH values, effect of catalase on compound 3. (: Compound solution in distilled water (0.1 mM), pH of the solution 5.2. × : Solution of complexes in phosphate buffer (pH 7.0). : Effect of catalase. The test solution contained phosphate buffer (50 mM, pH 7.0) and the catalase (40 μg /mL) which was incubated at 30°C for 5 min. Compounds (0.1 mM) were then added to the reaction solution and aliquots were drawn at indicated time points and loss in peroxide content was determined.

undergo multistage decomposition. The complexes do not explode on heating. The TGA curve of compound **3** shows the first stage of decomposition occurring between room temperature and temperature of 110°C with the liberation of the outer sphere water molecules from the complex. The corresponding observed weight loss of 10.3% is in good agreement with the calculated value of 11.5% for the loss of three molecules of water of crystallization. The next decomposition stage is a two step one in the temperature range 110–240°C with a corresponding weight loss of 17.45% attributable to loss of co-ordinated water and peroxo groups from the complexes, which agrees well with the calculated value of 17.55%. The results thus provided conclusive evidence for the presence of co-ordinated, as well as lattice water in the compounds. Absence of peroxo group in the decomposition product, isolated at this stage, was confirmed from the infrared spectral analysis. The loss of peroxide is seen to be followed by another decomposition step occurring in the temperature range of 240–465°C. The corresponding weight loss of 27.17% for this decomposition agrees well with the value of 28.05% calculated for the loss of coordinated ligand (gly-gly) from the complex. The residue remaining at this stage was found to be an oxo-tungsten species as indicated by the IR spectra, which displayed the characteristic ((W═O) absorption and was devoid of bands attributable to peroxo and the dipeptide ligands of the original compound. Thermogravimetric analysis data of the compound thus provided further evidence in support of the composition and formula assigned to the compounds.

*Stability of the complexes **1**–**4** in solution – their action with catalase*

The investigations on the stability of the compounds in an aqueous solution of pH ca. 5, which is the natural pH attained by the solution of the compounds **3** and **4** in water, revealed that their peroxide content and position and intensity of their electronic spectral bands remained unaltered for over a period of 12 hours. Figure 2 shows that the compound **3**, used as a representative, is stable in solution of pH 5.5 as well as at pH 7.0. We further examined and ascertained their stability in solutions of pH values ranging from 3.6 to 8.0. Stability of these monomeric compounds was found to be comparable to those of dimeric amino acid or peptide containing pW analogues [26].

In the context of the present study which mainly focus on investigating some biochemically relevant properties of the pW complexes, it was considered imperative to examine the sensitivity of the pW complexes **1**–**4** towards catalase, the enzyme that catalyze the breakdown of H_2O_2 formed during oxidative processes in the intercellular peroxisomes. On incubation with catalase, each of the monomeric and dimeric pW compounds was found to be degraded gradually with the loss of peroxide. The effect of catalase on the complexe **3** is shown in Figure 2. Total peroxide loss from each of the pW compound solution of 0.1 mM concentration tested was recorded to be ca. 0.4, and ca. 0.2 mM for dinuclear and mononuclear compounds, respectively (Table II) indicating a ratio of 1:4 for peroxide: dinuclear complex (compounds **1** and **2**) and 1:2 for mononuclear pW compounds (**3** and **4**) which is in excellent agreement with the estimated peroxide content of the compounds. The extent and initial rate of degradation of the dimeric complexes **1** and **2** under the effect of catalase action (Figure 2) were

Table II. Catalase-dependent oxygen release from peroxotungsten compounds **1**–**4**.

Compounds	Conc(mM)	Loss of peroxide	
		μM /min.	Total(μM)
$Na_2[W_2O_3(O_2)_4(gly\text{-}gly)_2].3H_2O$ (**1**)	0.1	21.3	39.2
$Na_2[W_2O_3(O_2)_4(gly\text{-}leu)_2].3H_2O$ (**2**)	0.1	24.1	39.4
$[WO(O_2)_2(gly\text{-}gly)(H_2O)].3H_2O$ (**3**)	0.1	6.8	19.1
$[WO(O_2)_2(gly\text{-}leu)(H_2O)].3H_2O$ (**4**)	0.1	8.7	19.7

found to be comparable to that of $A_2[W_2O_3(O_2)_4(-cystine)].4H_2O$ (ca. 20 μM/min from a solution of 0.2 mM) [25] indicating their similarity with respect to number of peroxide and pattern of their co-ordination to the W(VI) center. Under the effect of catalase the rate of degradation of H_2O_2 with the release of oxygen was reported to be 430 μM/min [39] from a solution of 0.1 mM concentration and the reaction will be completed in less than 2 min. Thus the rate of H_2O_2 degradation is several fold higher than the rate of degradation observed for the title compounds under similar reaction conditions. It is thus evident that the synthesized pW complexes are at least 20 times weaker as substrates to catalase compared to H_2O_2, its natural substrate.

*Oxidation of glutathione (GSH) by peroxo complexes **1–4***

Oxidizing capacity of peroxometallates with respect to reduced GSH, were tested by the method of Ellman [30]. The monomeric as well as dinuclear peroxo tungsten compounds efficiently oxidized GSH, to GSSG. Amounts of GSH oxidized at two different concentrations of 5 and 10 μM of the compounds **1–4**, are presented in Figure 3. If all the four peroxo groups of the dimeric compounds (**1** and **2**) were active in oxidation, 8 moles of GSH to GSSG per mole of the compound should have been oxidized. Similarly, in view of the presence of two peroxo groups it is expected that compounds **3** and **4** would oxidize 4 moles GSH. However, the consistently observed stoichiometry of 4: 1 for GSH oxidized: dinuclear pW compounds (**1** or **2**) and 2:1 for GSH: mononuclear pW compounds (**3** or **4**) cause us to infer that only one of the peroxo groups of a diperoxotungsten, $WO(O_2)_2$ moiety of the monomeric compounds or two of the peroxo groups of the oxo-bridged tetraperoxo tungsten species, $[\{WO(O_2)_2\}_2O]$ of compounds **1** and **2** would be active in GSH oxidation.

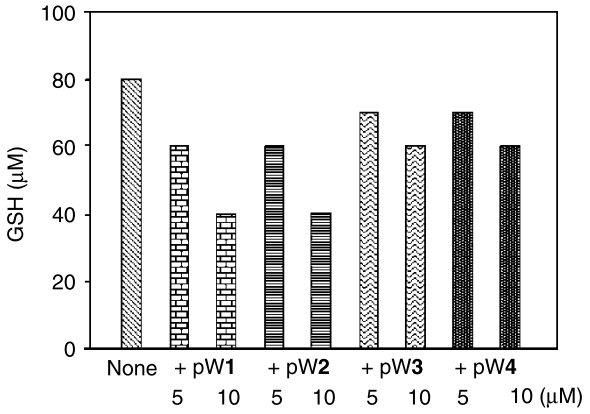

Figure 3. Oxidation of GSH by pW compounds **1- 4.** The reaction mixture contained GSH (80μM) and phosphate buffer (50 mM, pH 7.0). A measured amount of the synthesized compound was added. Measurements were done by using two different concentrations of the compounds, 5.0 and 10.0 μM in triplicate under same assay conditions. Following incubation of 10 min, DTNB (160 μM) was added to the solution and the change in absorbance at 412 nm was determined in order to measure the GSH remaining in solution.

*Effect of the compounds **1–4** on alkaline phosphatase activity*

Alkaline phosphatase is a membrane-bound zinc metalloenzyme with a broad substrate specificity, which catalyzes the hydrolysis of organic phosphate monoesters possibly via an enzyme-phosphate intermediate. The maximum activity of the enzyme is observed at pH ≥ 8. Phosphotransferase activity and protein phosphatase activity are some of the other probable functions assigned to the enzyme.

In the present study, we have examined the effect of different concentrations of the newly synthesized peroxo-metal complexes upon ALP activity of rabbit intestine alkaline phophatase employing established enzyme assay system and p-NPP as substrate. The dose dependent effects of the mononuclear and dinuclear peroxo tungstate complexes **1–4** compared with the free ligands, W(VI), W(VI)- H_2O_2 systems, respectively are presented in Figure 4. We determined the half-maximal inhibitory concentration (IC_{50}) for each inhibitor, which gave rise to a 50% suppression of the original enzyme activity (Table III) in order to quantify the inhibitory potential of the molecules. The enzymatic rate ratios V_0/Vi where V_0 is the uninhibited rate and Vi is the rate of the enzymatic reaction inhibited by the complexes and other species were found to be directly proportional to the different compound concentrations (Figure 5).

Discussion

Tungsten has strong affinity for peroxide and the composition of peroxo-tungsten species has been known to be sensitive to pH [4,31,32]. In a solution of tungstate and excess H_2O_2 at a pH ≥ 5 diperoxotungstate species is readily formed, whereas the dimeric species, $[W_2O_3(O_2)_4]^{2-}$ predominates at pH ≤ 5 [4,31,32]. Thus in the present study, the strategically maintained pH of ca. 5 was found to be conducive for the successful synthesis of the molecular compounds **3** and **4**. It is notable that maintenance of acidic pH of ca. 3 was one of the essential requirements for achieving syntheses of the amino acid or peptide contaning oxo-bridged tetraperoxotungstates **1** and **2** [25,26].

The dipeptide ligands contain several potential donor sites and as such, depending on the metal ion, pH and solution composition, can lead to a variety of co-ordination modes involving the terminal amino, carboxylate groups as well as amide linkage [33,40]. Thus the chosen ligands can act as mono, bi or

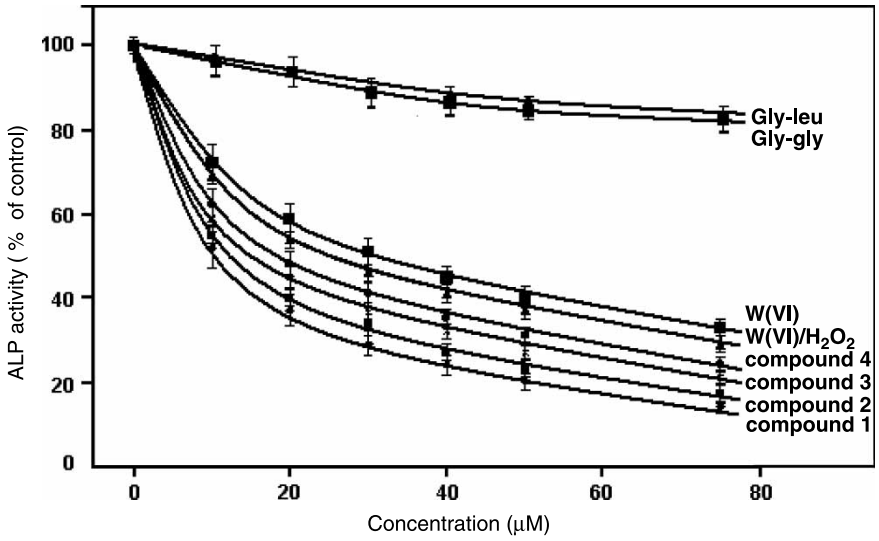

Figure 4. Effect of compounds **1 - 4**, gly-gly, gly-leu, W (VI) and W (VI)/H_2O_2 species on activity of ALP from rabbit intestine. The ALP catalyzed rates of hydrolysis of *p*-NPP at pH 10.0 were determined at 30 ^{0}C by measuring A_{405} in a reaction mixture containing ALP (3.3 μg/mL), *p*-NPP (1 mM) in incubation buffer (25 mM glycine +2 mM $MgCl_2$, pH 10.0) in the absence or presence of stated concentrations of the inhibitors. Effects of the additions are represented as the percent values (rounded to integers) of control (Δp-NP = 0.96 μM/min). The data are presented as the means ± SE from three separate experiments.

tridentate species and can occur in complexes in either neutral zwitterionic form or anionic form. In the present case, the pH ca. 5 of the reaction medium is unlikely to allow deprotonation of the amide group of the peptide and thereby limits co-ordination to the metal centres through carboxylate group of the peptide zwitterions. Although the bi or tridentate co-ordination is more common in amino acid or peptide containing complexes however, monodentate co-ordination through a carboxylate group of the aminoacid or peptide zwitterion is not unprecedented [26,41–43]. The high stability of the compounds **1**–**4** in solution at acidic as well as physiological pH is likely to be due to the additional stability imparted by the ancillary ligands.

We have been particularly interested to investigate the interaction of the synthesized complexes with glutathione (GSH) in view of the literature report where it has been demonstrated that [10] the higher efficacy of the pertungstates and permolybdates as insulinomimetic agents is a consequence of their oxidizing activity relative to glutathione (GSH). Glutathione is the major non protein thiol in living cells, which plays the role of cellular reducing agents and antioxidant [44]. It was intriguing to note that, although the compounds **1**–**4** undergo degradation under the effect of catalase with total loss of peroxide, the oxidation activity of the pW compounds tested irrespective of being monomeric or dimeric, was limited to ca. 50% of that expected on the basis of the total number of peroxo groups present in these complexes. Similar observation was made earlier while investigating the GSH oxidizing ability of the complex species, $[W_2O_3(O_2)_4(\text{cystine})]^{2-}$ [25]. The result of the investigation is consistent with the proposal implicating formation of a monoperoxo-W (VI) intermediate, which is inactive in GSH oxidation and is in accord with the earlier suggestions that for a peroxotungsten complex to be active in oxidation an oxo-diperoxo configuration may be a prerequisite [6]. Significantly, during one of our previous studies involving redox activity of the complexes **1** and **2** in oxidative bromination, only two of the peroxo groups out of 4 peroxides present per molecule of the complex were found to be involved in bromide oxidation [26]. However, there appears to be no plausible explanation as to why only half of the peroxide groups present per molecule of a pW complex should participate in oxidation.

The most noteworthy feature emerging out of our data derived from Vo/Vi relationships and IC_{50} values

Table III. Half-maximal inhibitory concentration (IC_{50}) values of the compounds **1–4** and other inhibitors against ALP.

Inhibitor	IC_{50} (μM)
$Na_2[W_2O_3(O_2)_4(\text{gly-gly})_2].3H_2O$ (**1**)	10.29
$Na_2[W_2O_3(O_2)_4(\text{gly-leu})_2].3H_2O$ (**2**)	12.67
$[WO(O_2)_2(\text{gly-gly})(H_2O)].3H_2O$ (**3**)	15.84
$[WO(O_2)_2(\text{gly-leu})(H_2O)].3H_2O$(**4**)	19.80
Tungstate	31.68
Tungstate/H_2O_2	25.34
Glycyl-glycine	–
Glycyl-leucine	–

Note: The ALP catalyzed rates of hydrolysis of *p*-NPP at pH 10.0 were determined at 30°C by measuring A_{405} in a reaction mixture containing ALP (3.3 μg/mL), p-NPP (1 mM) in incubation buffer (25 mM glycine +2 mM $MgCl_2$, pH 10.0) in the presence of stated concentrations of the inhibitors (Figure 4).

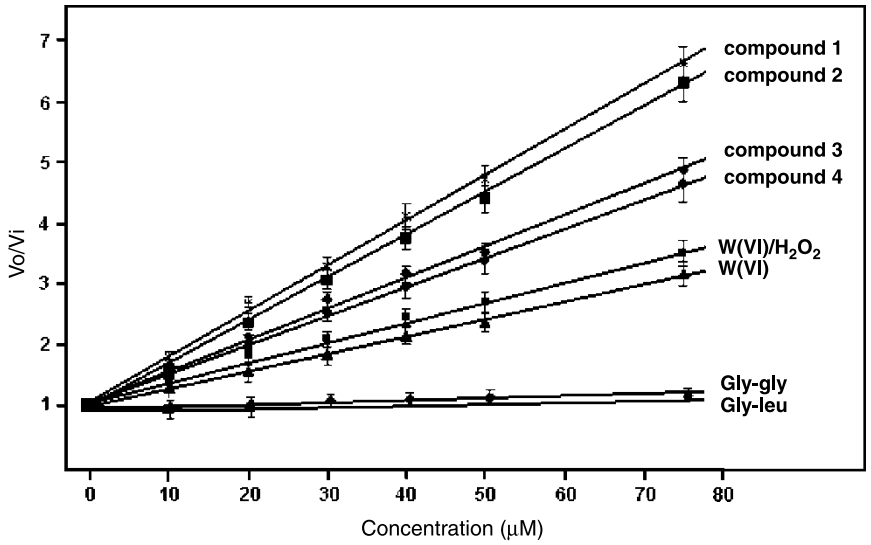

Figure 5. V_0/V_i ratios for the inhibition of compounds **1 - 4,** gly-gly, gly-leu, W (VI) and W(VI)/H_2O_2 species in the alkaline phosphatase catalyzed hydrolysis of *p*-NPP. The reaction mixture contained glycine buffer (25 mM glycine + 2 mM $MgCl_2$, pH 10.0) and p-NPP (1 mM). The reaction was started by adding ALP (3.3 μg/mL) to the reaction solution which was pre-incubated for 5 min and the rate of hydrolysis in the absence of the inhibitors, V_0, was obtained. The inhibited rates of hydrolysis, V_i, were determined as above and in the presence of stated concentrations of inhibitors. The values are expressed as means ± SE from three separate experiments.

is that although individually each of the tested species inhibited ALP activity to varying degrees, the peptide containing peroxotungsten complexes turned out to be the most effective inhibitors. Complexes with gly-gly as ancillary ligand are more potent inhibitors than the corresponding gly-leu containing analogue. Further, among the dimeric pW compounds **1** and **2** and previously tested compound $A_2[W_2O_3(O_2)_4$ (cystine)]. $4H_2O$ with an IC_{50} value of ca. 8.0 μM [25], the following trend in inhibitor potency has been noted: $[W_2O_3(O_2)_4(\text{cystine})]^{2-}$ > compound **1** > compound **2**. Previous studies indicated that phosphatases are, in general, inhibited by oxyanions such as vanadate [22–24], molybdate and tungstate [9,13]. Such inhibition has been attributed to the formation of pentaco-ordinated or hexaco-ordinated structures, which are described as phosphate analogues [9,13,22–24]. In the present study, it is notable that the inhibitor potency of the compounds tested, despite having hepta-coordinated metal center in each of them, is much higher than expected from equivalent concentrations of tungstate or peroxotungstate formed in solution. In this context it is also appropriate to recall the findings of Crans *et al.* that some six- or seven-coordinate vanadium or pV compounds inhibit phosphatases, however, five coordinated compounds are more potent inhibitors than the 6 or 7 co-ordinated ones [24]. The above observations demonstrate that structural analogy with the transition state or phosphate mimicry may not be the only factor due to which metal complexes may exert inhibitory effect on protein phosphtases. Although it is evident from our data that the inhibitor potencies of the compounds are sensitive to the nature of co-ligand environment, however, the possibility of the observed inhibition being caused by the amino acid or peptide co-ligand alone may be ruled out since the effect of individual ligands on ALP activity is practically negligible under the assay conditions used and H_2O_2 as such had no observable effect. In absence of direct evidence at this stage we are unable to discern definitive reasons for the effect of the title compounds on the phosphatase activity. On the basis of our observation made on of the pW complexes [25] in conjunction with the reports documenting the importance of redox properties of peroxo vanadium compounds in inhibition of protein phosphatases [22–24], we have previously proposed the oxidant properties of the title complexes as one of the possible factors responsible for making the compounds effective inhibitors of the phospho proteins [25]. Results of the present investigation lend further support to this proposal. Correlation has also been reported to exist between the oxidizing ability and insulin mimetic activity of peroxo compounds of tungsten and molybdenum [10].

Conclusions

The newly synthesized compounds represent a set of water soluble peroxo derivatives which contain species familiar to bioenvironment as ancillary ligand. The present experiments confirm that the pW compounds **1**–**4**, irrespective of being monomeric or dimeric, are potent inhibitors of ALP activity. An additional distinctive feature of the compounds, which may be of clinical relevance, is their high stability in solution at a wide range of pH values, particularly at acidic pH.

The compounds thus fulfill one of the criteria for metal complexes to be useful as therapeutic agent and provide future scope for testing such properties. It is also noteworthy that the compounds are relatively resistant to degradation by the powerful enzyme catalase and utilise their peroxide groups only partially during interaction with GSH. This may be relevant in the cellular milieu where H_2O_2 has little chance to survive abundant catalase and glutathione peroxidase.

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