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Mn(III) oxidation of peptide polymers of elastin sequences: synthesis, kinetics and mechanistic study

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ABSTRACT: Protein based-polymers comprised of Ala-, Val- and Ile-containing tetrapeptide repeating sequences of elastin were synthesized based on the increasing order of hydrophobicity and subjected to metal-catalyzed oxidation to identify the amino acid residues, which are sensitive to oxidation. These polytetrapeptides (PTP) were oxidized using $Mn(OAc)_3$ at 25 °C and the kinetics of the reaction were monitored spectrophotometrically at $\lambda_{max} = 400$ nm. A first-order rate dependence on the substrate concentration [PTP] and [Mn(III)] and an inverse order dependence on $[H^+]$ has been observed. Further, the rate is independent of $[Mn(OAc)_2]$. The effect of the dielectric constant on the rate was studied by varying the percentage of AcOH. Activation parameters were evaluated using Arrhenius and Eyring plots. The oxidation products were isolated and characterized. Based on the results obtained, a plausible mechanism involving $[Mn(OAc)_4]^-$ is proposed. It is noteworthy that the rate of oxidation of poly(GGIP) was higher than those of poly(GGVP) and poly(GGAP). This may be due to their increased hydrophobicity. The overall order of rate of oxidation of PTP is poly(GGIP) > poly(GGVP) > poly(GGAP). Copyright © 2005 John Wiley & Sons, Ltd.

INTRODUCTION

Metal-catalyzed oxidation reactions of cholesterol, lipids, carbohydrates, DNA, RNA, proteins and antioxidants play an important role in biological processes.[1–4] In particular, spontaneous changes in protein structure due to its oxidative modifications with reactive oxygen species is an important event in age-related alterations, oxidative stress and some pathological conditions. The changes in protein conformations due to oxidative damage are often related to changes in the hydrophobicity of the protein sequences. This is the phenomenon associated with the physiological conditions of protein modifications and is necessary to understand the mechanism involved in biological processes. Hydrophobic moieties of protein are considered to be more susceptible to oxidation.

Therefore, the protein-based polymers of elastin were taken as a model system to identify the amino acid residues, which are sensitive to metal-catalyzed oxidation. Protein-based polymers are polypeptides comprised of repeating sequences of amino acids, having their origin in a protein, elastin. The most striking repeating sequence $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ or $(VPGVG)_n$ is apparent in the bovine and porcine elastins[5,6] and another repeat $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4)_n$, was first found in porcine elastin.[7] The monomers, oligomers and high molecular weight polymers of these repeats have been synthesized and conformationally characterized.[8] Such protein-based polymers exhibit the same hierarchical structure and mimic the parent protein. These polymers have a number of medical and non-medical applications.[9,10] The hydrogel state of poly(GGAP) or $(Gly\text{-}Gly\text{-}Ala\text{-}Pro)_n$ is most extraordinarily biocompatible and non-mutagenic, which is suitable for the controlled release of drugs.[11] The cross-linked polytetrapeptide matrices based on the repeating amino acid sequences Gly-Gly-Ala-Pro, Gly-Gly-Ile-Pro and Gly-Gly-Val-Pro were tested for cell adhesion-promoting activity in both the absence and presence of fetal bovine serum.[12] The degree of cell attachment increased with increase in hydrophobicity. Therefore, in particular, hydrophobicity and hydrophilicity are the two most important phenomenon of interest exhibited by the peptides and proteins. In this context, interest was generated in investigating the oxidative behavior of these analogues of repeating sequences of elastin with metal ions.

Extensive work has been reported on the kinetics of oxidation of proteins and its constituents with various oxidants including metal ions.[13,14] Manganese compounds have attracted much attention with regard to the oxidation of various biological substrates. Mn(III) porphyrins have been studied as possible models for closely related biologically significant systems.[15] In this respect, Mn(III) oxidation of protein-based polymers is gaining special importance owing to its biological relevance. In recent years, the kinetics of oxidation of amino acids, their derivatives and peptides have been studied using Mn(III) as an oxidizing agent in different media.[16–21] This is the first attempt at kinetic and

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mechanistic investigations of the oxidation of protein-based polymers of elastin sequences.

EXPERIMENTAL

General

All the amino acids used were of L-configuration unless indicated otherwise. All Boc-amino acids, 1-ethyl-3-(*N*,*N*-dimethyl)aminopropylcarbodiimide (EDCI), 1-hydroxybenzotriazole (HOBt) and trifluoroacetic acid (TFA) were purchased from Advanced Chem Tech (Louisville, KY, USA). *N*-Methylmorpholine (NMM) was procured from Sigma Chemical (St. Louis, MO, USA). Mn(III) acetate, acetic acid and sodium acetate were obtained from E. Merck (India), (Mumbai, India). All solvents and reagents were of analytical grade or were purified according to procedures recommended for peptide synthesis.

Peptide synthesis and polymerization

Synthesis of Gly-Gly-Xaa-Pro (Xaa = Val, Ala or Ile). Boc-Gly-Gly-Val-Pro-OBzl, Boc-Gly-Gly-Ala-Pro-OBzl and Boc-Gly-Gly-Ile-Pro-OBzl were synthesized as described elsewhere.[19] They were then hydrogenated to the corresponding Boc-Gly-Gly-Xaa-Pro-OH using Mg–HCO_2 HNH_2NH_2 as described earlier.[22] The Boc-Gly-Gly-Xaa-Pro-OH (5 mmol) were subjected to deblocking separately with TFA (10 ml g^{-1} of monomer) to obtain the corresponding TFA salt of Gly-Gly-Xaa-Pro.

Synthesis of poly(Gly-Gly-Xaa-Pro). Solutions of 1 M TFA salts in DMSO were polymerized separately for 2 days using EDCI (2 equiv.) with HOBt (1 equiv.) and NMM (1.6 equiv.) as base.[23] The polymers were dissolved in water, dialyzed using 3500 Da molecular weight cut-off dialysis tubing for 1 week and lyophilized. They were then dialyzed using 50 kDa molecular weight cut-off dialysis tubing for 1 week and lyophilized.

Preparation of Mn(III) acetate

A 0.02 M solution of $Mn(OAc)_3$ was prepared by dissolving it in 25% (v/v) acetic acid. To this solution, a 0.5 M solution of sodium acetate was added. Addition of sodium acetate enhances the solubility of $Mn(OAc)_3$ owing to the formation of an acetato complex $[Mn(OAc)_4]^-$ and it facilitates the increase in rate of reaction. Even though the solution was found to be stable for > 2 days at $[H^+] > 5.0$ M, it was prepared fresh daily.

Preliminary studies

The absorption spectra of freshly prepared $Mn(OAc)_3$ solution was recorded using an Analytic Jena Specord 50 spectrophotometer with quartz cells of 1 cm pathlength. The visible spectra of $Mn(OAc)_3$ solution showed λ_{max} at 440 nm. However, when sodium acetate was added to $Mn(OAc)_3$, λ_{max} was shifted to 400 nm, owing to the formation of an acetato complex $[Mn(OAc)_4]^-$. This is in accordance with the observation made by Midgley and Thomas.[24] The standard reduction potential E'_0, the oxidizing power of the oxidant Mn(III)/Mn(II), generally decreases on complexation. The standard redox potential was also measured under the specified experimental conditions and the results were found to be identical with those in the previous report.[25] The formal redox potential E'_0 obtained at different concentrations of acetic acid is 1.160 V for the pure system and in the presence of other complexing agents such as ClO_4^-, Cl^-, $P_2O_7^{4-}$ and HSO_4^-, in the form of sodium salts, the formal redox potential E'_0 are 1.169, 1.181, 1.48 and 1.51 V, respectively.

Kinetic measurements

The solutions of PTP, sodium acetate and water were prepared in separate stoppered boiling tubes. They were thermally equilibrated in a water-bath at 25 °C. From these solutions, a mixture of known concentrations of PTP (calculated based on the monomeric tetrapeptide unit), sodium acetate (to maintain constant ionic strength) and water (to keep the total volume constant) was prepared. To this mixture was added an aliquot of pre-equilibrated $Mn(OAc)_3$ stock solution to give a known overall concentration. The progress of the reaction was monitored for two half-lives (for about 1.5 h with a 5 min interval) by measuring the absorbance of unreacted Mn(III) at λ_{max} 400 nm. Plots of log(absorbance) versus time were linear. The rate constants k_{obs} calculated from these plots were reproducible to within $\pm 3\%$ error.

RESULTS AND DISCUSSION

From both the NMR spectra of polypeptides and the HPLC traces from amino acid analysis, the absence of extraneous peaks verified the synthesis and the ratio of relevant peaks provides the ratios of amino acid residues in the polymers as depicted in Table 1.

The kinetics of oxidation of PTP were studied by varying the concentration of Mn(III) at a constant temperature of 25 °C (Table 2). Plots of log[Mn(III)] versus time were linear even beyond 75% reaction, showing a first-order dependence of the rate on [Mn(III)]. At constant $[Mn(III)]_0$, $[Mn(II)]_0$, $[NaOAc]_0$ and temperature, the rate increased with increase in [PTP] (Table 2). Plots of $\log k_{obs}$ versus log[PTP] are depicted in Fig. 1. The plots were linear with slopes of 0.92, 0.98 and 1.10 for poly(Gly-Gly-Ala-Pro), poly(Gly-Gly-Val-Pro) and poly(Gly-Gly-Ile-Pro), respectively.

Table 1. Amino acid composition of free peptides[a]

Peptide	Gly	Pro[b]	Ala	Val	Ile
Poly(GGAP)	2.02 (2.00)	1.00	0.91 (1.00)	—	—
Poly(GGVP)	1.98 (2.00)	1.00	—	0.99 (1.00)	—
Poly(GGIP)	2.08 (2.00)	1.00	—	—	0.98 (1.00)

[a] Theoretical values are shown in parentheses.
[b] Pro residue was taken as 1.00.

Table 2. Effect of varying reactant concentration on the reaction rate in 25% acetic acid medium at 25 °C

$[Mn(III)] \times 10^{-3}$ (mol l^{-1})	$[PTP] \times 10^{-2}$ (mol l^{-1})	$[NaOAc] \times 10^{-1}$ (mol l^{-1})	$k_{obs} \times 10^5$ (s^{-1})		
			Poly(GGAP)	Poly(GGVP)	Poly(GGIP)
0.4	1.0	5.0	7.06	9.25	12.36
0.7	1.0	5.0	7.32	9.47	12.41
1.0	1.0	5.0	7.11	9.81	12.46
1.3	1.0	5.0	7.36	9.92	12.85
1.6	1.0	5.0	7.82	9.31	12.57
1.9	1.0	5.0	7.34	9.67	12.68
2.2	1.0	5.0	7.61	9.55	12.78
1.0	0.6	5.0	5.29	7.06	10.91
1.0	0.8	5.0	6.64	8.57	11.57
1.0	1.0	5.0	7.12	9.81	12.46
1.0	1.2	5.0	9.27	10.91	14.25
1.0	1.4	5.0	10.53	11.89	15.85
1.0	1.6	5.0	11.43	12.73	17.02
1.0	1.8	5.0	12.83	14.40	18.28
1.0	2.0	2.5	14.35	16.98	19.85
1.0	1.0	0.0	7.12	9.81	11.46
1.0	1.0	5.0	8.94	10.12	12.12
1.0	1.0	10.0	9.35	10.98	13.95
1.0	1.0	15.0	9.89	11.73	14.65
1.0	1.0	20.0	10.93	12.23	15.25
1.0	1.0	25.0	11.52	13.38	16.85

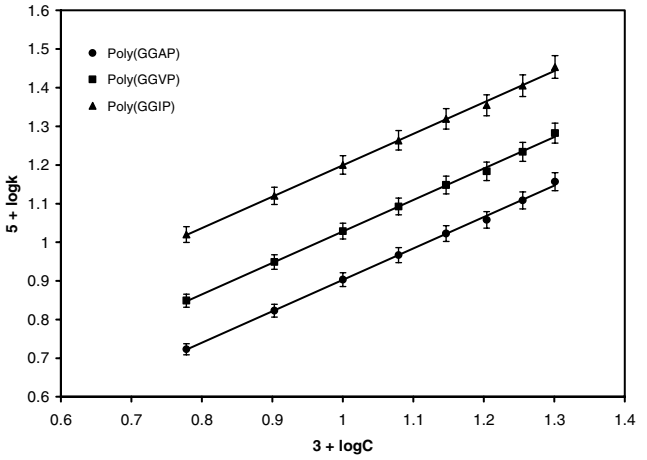


Figure 1. Effect of [PTP] on the rate at 25 °C, $[Mn(III)] = 1 \times 10^{-3}$ mol l^{-1} and $[NaOAc] = 0.5$ mol l^{-1}

The effect of concentration of Mn(II), a reaction product, on the rate was investigated. Sequential increase of [Mn(II)] from 0.01 to 0.1 M had no effect on the rate, indicating that the product is not involved in a pre-equilibrium with the oxidant. Similarly the effect of anion OAc^- increases the rate with increase in [NaOAc] (Table 2), but an increase in the concentrations of other anions such as Cl^- (from 0.01 to 0.5 M) and ClO_4^- (from 0.01 to 1.0 M) has an insignificant effect on the rate. The effect of the dielectric constant (D) on the rate was studied by varying the percentage of AcOH (Table 3). The rate decreased with increase in AcOH content. Plots of k_{obs} versus $1/D$ are presented in Fig. 2 and are linear with a negative slope, in agreement with the Amis concept of dipole–dipole and ion–dipole interactions.[26]

To determine the activation parameters, the reactions were carried out at different temperatures (25–40 °C). As poly(GGIP) was insoluble at higher temperatures, the thermodynamic parameters were not studied. The Arrhenius plots of log k_{obs} versus $1/T$ (Figure 3) were found to be linear. The activation energies (E_a) were calculated from the different temperatures. From this mean value, the thermodynamic parameters $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, $\Delta G^{\ddagger}$ and the frequency factor (log$A$) for poly(GGAP) and poly(GGVP) were evaluated and found to be $E_a = 37.15$, $34.25\,kJ\,mol^{-1}$; $\Delta H^{\ddagger} = 34.65$, $31.74\,kJ\,mol^{-1}$; $\Delta S^{\ddagger} = -207.52$, $-215.63\,kJ^{-1}$ mol^{-1}; $\Delta G^{\ddagger} = 98.88$, $96.80\,kJ\,mol^{-1}$; and $\log A = 2.40$, 1.97,

Table 3. Effect of varying dielectric constant on the reaction rate at 25 °C, $[Mn(III)] = 1.0 \times 10^{-3}$ mol l^{-1}, $[PTP] = 1.0 \times 10^{-2}$ mol l^{-1} and $[NaOAc] = 0.5$ mol l^{-1}

(%,v/v) [AcOH]	Dielectric constant, D (D)	$1/D \times 10^{-3}$	$k_{obs} \times 10^5$ (s^{-1})		
			Poly(GGAP)	Poly(GGVP)	Poly(GGIP)
15	68.1	14.6	8.325	10.592	13.184
25	60.9	16.4	7.265	9.811	12.456
35	53.7	18.6	6.302	9.291	9.564
45	46.5	21.5	5.793	8.021	8.645
55	39.3	25.4	5.553	7.032	7.245

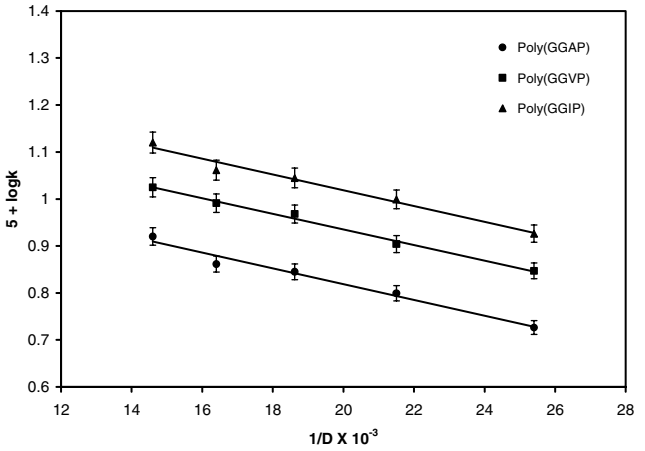


Figure 2. Effect of dielectric constant on the rate at 25 °C, $[Mn(III)] = 10 \times 10^{-3}$ mol l^{-1}, $[PTP] = 10 \times 10^{-3}$ mol l^{-1} and $[NaOAc] = 0.5$ mol l^{-1}

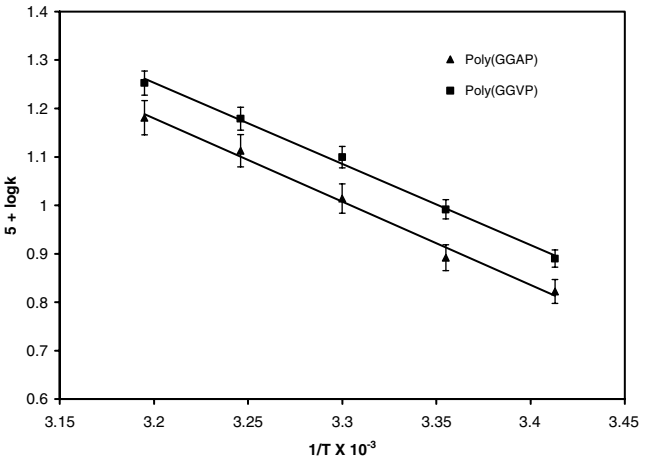


Figure 3. Plot of k_{obs} versus $1/T$ at $[Mn(III)] = 10 \times 10^{-3}$ mol l^{-1}, $[PTP] = 10 \times 10^{-3}$ mol l^{-1}, $[NaOAc] = 0.5$ mol l^{-1} and different temperatures

respectively. The negative entropy of activation ($\Delta S^{\ddagger}$) for all the PTP indicates that the involvement of a rigid associated species in the transition state, which is probably formed due to solvation lowering the entropy of activation. In addition, the positive values for $\Delta H^{\ddagger}$ and $\Delta G^{\ddagger}$ indicate that PTP react with Mn(III) via the proposed mechanism.

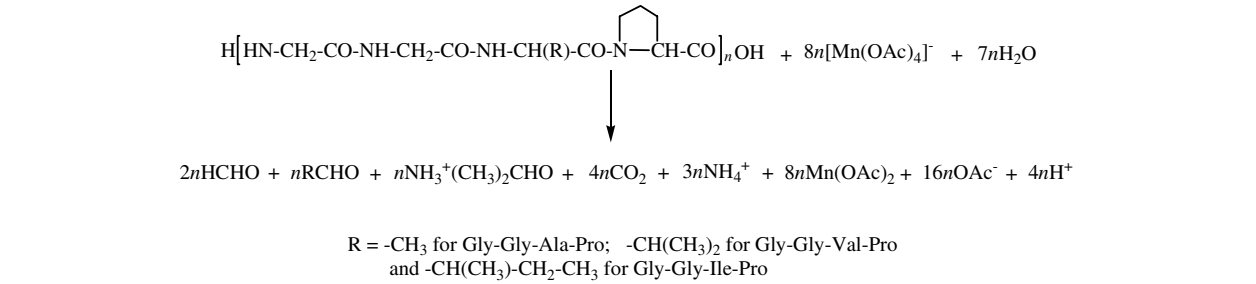
Addition of the reaction mixture to aqueous acrylamide–PTP solution initiates polymerization, indicating the *in situ* formation of free radical species during the reaction sequence.

Reaction stoichiometry and product analysis

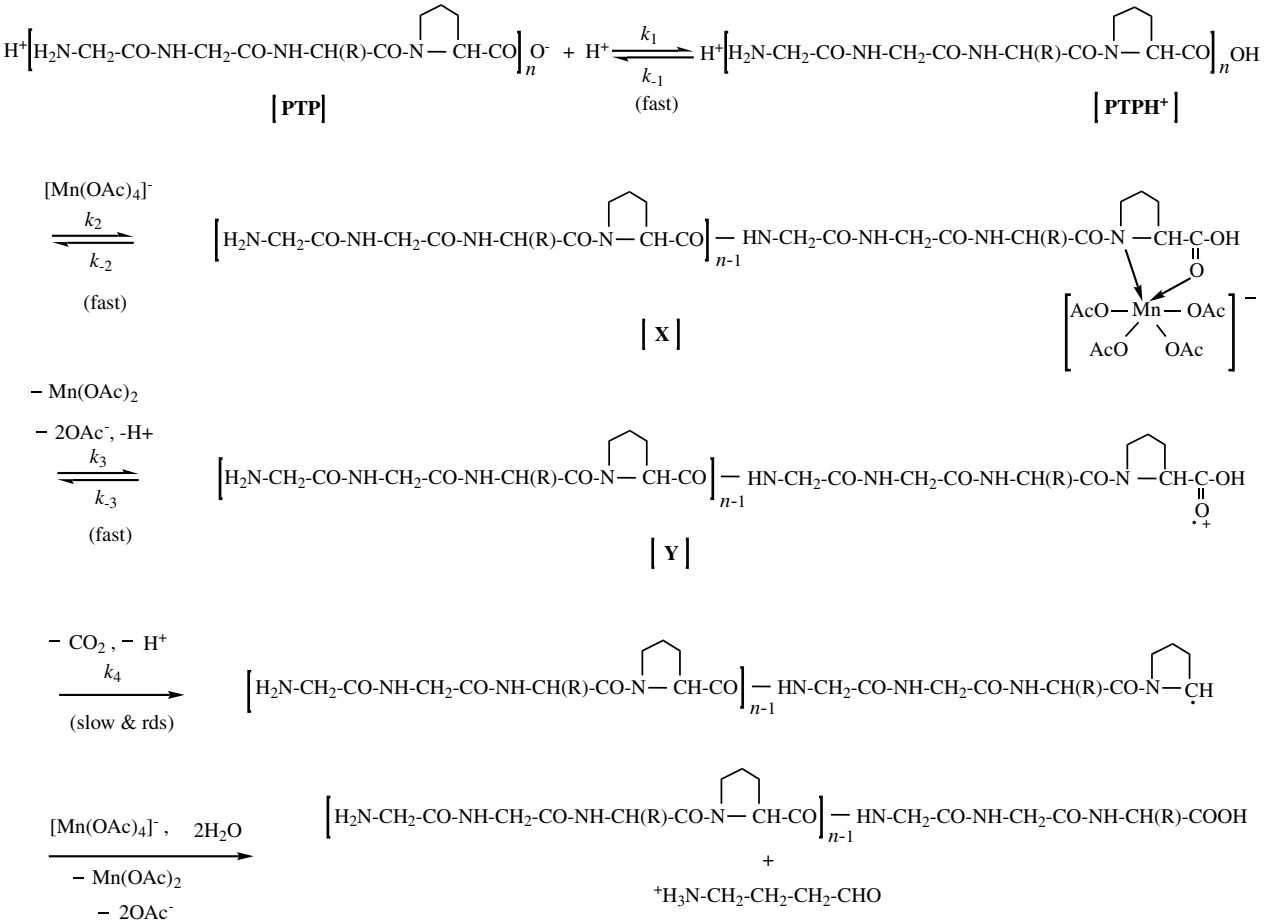
The mixtures containing PTP (0.001 M), NaOAc (0.5 M) and excess Mn(III) (0.01 M) were kept for 24 h at 25 °C. The unconsumed Mn(III) was then determined by the iodimetric method. Eight moles of oxidant are sufficient to oxidize 1 mol of PTP to produce aldehydes, carbon dioxide, ammonia, $Mn(OAc)_2$, OAc^- and hydrogen ion. After the completion of the reaction, the reaction mixture was quenched by pouring it into ice-cold water (50 ml). The products were extracted with diethyl ether and subjected to column chromatography on silica gel (60–200 mesh) using gradient elution (dichloromethane to chloroform). Aldehydes were analyzed qualitatively by gas chromatography. The retention times of formaldehyde, acetaldehyde, isobutyraldehyde, 2-methylbutyraldehyde and 4-aminobutyraldehyde were found to be 5.13, 5.92, 27.4, 31.1 and 32.1 min, respectively, and were identical with those of authentic samples. Ammonia and CO_2 were detected by the conventional tests. Based on these results the stoichiometric equations shown in Scheme 1 are suggested.

It is clearly shown from the previous studies that, in the presence of F^- ion, aqueous solution of Mn(III) consist of hexaaquamanganese(III)$\{[Mn(H_2O)_6]^{3+}\}$, $Mn(III)_{(aq)}$, hydroxopentaaquamanganese(II) $\{[Mn(OH)(H_2O)_5]^{2+}\}$, $Mn(OH)^{2+}_{(aq)}$ and $MnF^{2+}_{(aq)}$. In sulfuric acid medium, Mn(III) species present in acidic solution are $Mn(III)_{(aq)}$, $Mn(OH)^{2+}_{(aq)}$ and $MnSO^{4+}$.[19] However, in the present work, the $Mn(OAc)_3$ in acetic acid medium differ from others with respect to the oxidation of PTP, which involves the trivalent manganese, $[Mn(OAc)_3]$, or $[Mn(OAc)_4]^-$ as reaction species:

$$Mn(OAc)_3 + OAc^- \rightleftharpoons [Mn(OAc)_4]^- \qquad (1)$$



Scheme 1



Scheme 2

Scheme 2 accounts for the observed experimental results.

$$\text{Since, rate} = \frac{\mathrm{d}[\mathrm{Mn(OAc)_4^-}]}{\mathrm{d}t} = k_4[Y] \qquad (2)$$

Applying the steady-state approximation to intermediate species X and Y and by subsequent substitution of the results in Eqn (2), we obtain

$$\text{rate} = \frac{k_2k_3k_4[\mathrm{Mn(OAc)_4^-}][\mathrm{PTPH^+}]}{k_{-2}k_{-3}[\mathrm{Mn(OAc)_2}][\mathrm{H^+}][\mathrm{OAc^-}]^2 + k_{-2}k_4 + k_3\,k_4} \qquad (3)$$

Assuming that $k_{-2}k_4 \ll k_3k_4$ in the denominator

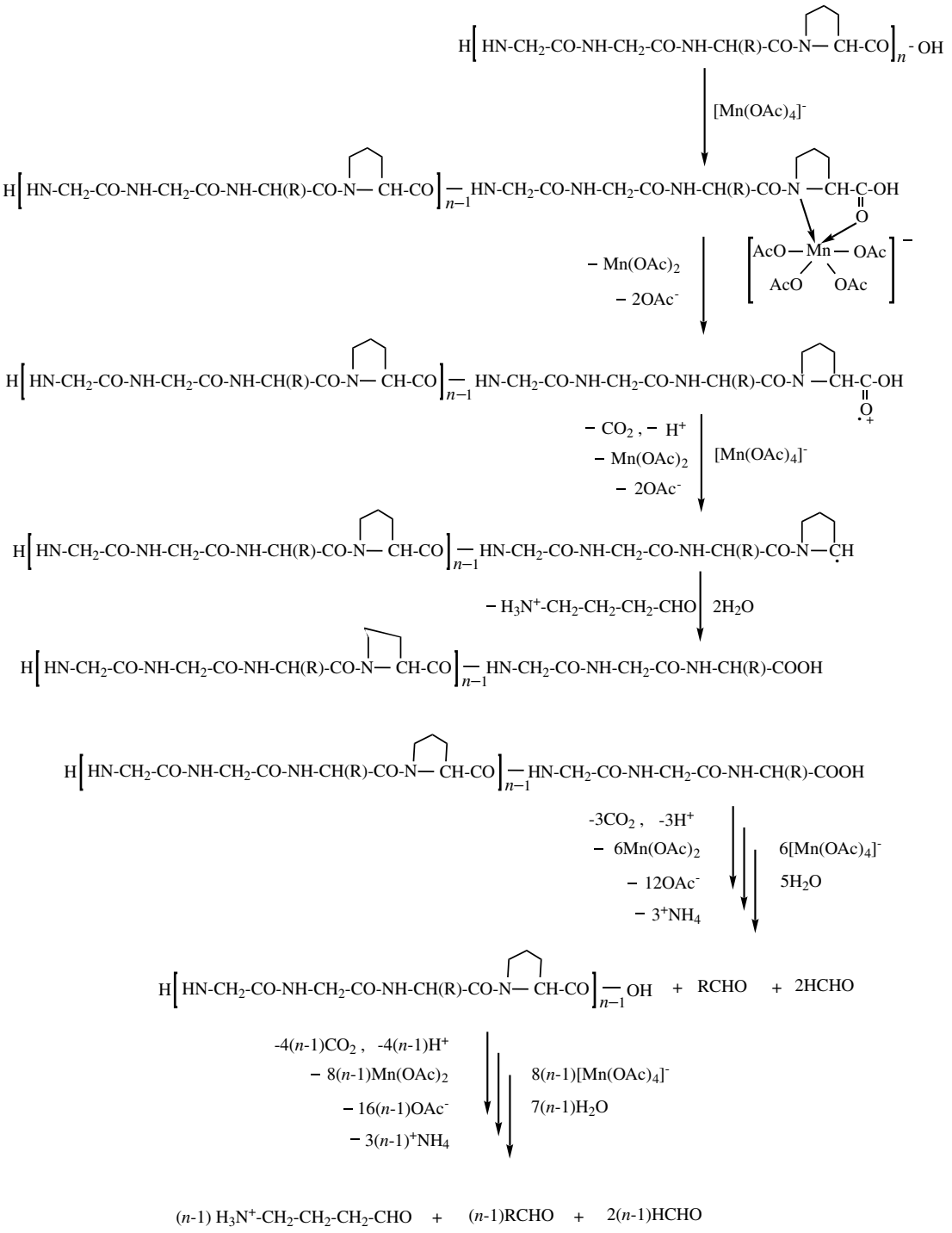
$$\text{rate} = \frac{k_2k_3k_4[\mathrm{Mn(OAc)_4^-}][\mathrm{PTPH^+}]}{k_{-2}k_{-3}[\mathrm{Mn(OAc)_2}][\mathrm{H^+}][\mathrm{OAc^-}]^2 + k_3\,k_4} \qquad (4)$$

$$\text{rate} = \frac{k_1k_2k_3k_4[\mathrm{Mn(OAc)_4}]^-[\mathrm{PTP}][\mathrm{H^+}]}{k_{-2}k_{-3}[\mathrm{Mn(OAc)_2}][\mathrm{H^+}][\mathrm{OAc^-}]^2 + k_3\,k_4} \qquad (5)$$

$$\text{rate} = \frac{k[\mathrm{Mn(OAc)_3}][\mathrm{OAc^-}][\mathrm{PTP}][\mathrm{H^+}]}{k_{-2}k_{-3}[\mathrm{Mn(OAc)_2}][\mathrm{H^+}][\mathrm{OAc^-}]^2 + k_3\,k_4} \qquad (6)$$

where $k = k_1k_2k_3k_4$.

Mechanism:



Scheme 3

The observed kinetic results shows a first-order dependence on [Mn(III)] and [PTP] and an inverse-order dependence on [H^+]. Addition of sodium acetate enhances the rate of reaction through the formation of active species, viz. the acetate complex $Mn(OAc)_4^-$. Therefore, the complex $Mn(OAc)_4^-$ can be considered as the kinetically active species involved in the oxidation of the PTP. The theoretical rate law is

Table 4. UV–visible spectral data for Mn(III), PTP and complex of Mn(III) + PTP

Substrate	λ_{max} (nm)	Absorbance	Complex	λ_{max} (nm)	Absorbance
Mn(III)	400.0	1.202			
Poly(GGAP)	214.5	3.712	Mn(III) + poly(GGAP)	219.2	3.729
Poly(GGVP)	216.2	3.730	Mn(III) + poly(GGVP)	218.4	3.739
Poly(GGIP)	216.8	3.756	Mn(III) + poly(GGIP)	218.8	3.760

$$\text{rate} = k[\text{Mn(OAc)}_3][\text{OAc}^-][\text{PTP}][\text{H}^+]/ \\ k_{-2}k_{-3}[\text{Mn(OAc)}_2][\text{OAc}^-]^2[\text{H}^+] + k_3k_4 \quad (7)$$

Based on these results and literature studies, a probable mechanism is proposed for the oxidation of PTP (Scheme 3). In the proposed mechanism, the formation of product, the aldehyde, is envisaged as due to the reaction of the intermediate PTP free radical with $Mn(OAc)_4^-$. A similar mechanism has been proposed for the Mn(III) oxidation of amino acids in aqueous acetic acid medium.[21]

The rate of oxidation of PTP by Mn(III) was found to be slower compared with tetrapeptides such as GGAP, GGVP and GGIP and tripeptides such as GAP, GVP and GIP, dipeptides such as AP, VP and IP and free amino acids in the presence of different aqueous acidic media.[16–19] The change in each case is due to the increased distance between the functional groups and consequently weaker electrostatic effects. In addition, reactive species induced by manganese not only oxidize amino acid residues in protein-based polymers, but also release peptide fragments and free amino acids. These released peptides and amino acids may also be the target of manganese induced reactive species. Hence the oxidation of the polytetrapeptides was expected to be slower than that of the amino acids, dipeptides, tripeptides and tetrapeptides. Based on these and other results discussed earlier, the most probable mechanism is that proposed in Scheme 3.

An apparent correlation was noted between the rate of oxidation and the hydrophobicity of these sequences, where increased hydrophobicity results in increased rate of oxidation. Therefore, in the PTP, the more hydrophobic Ile amino acid residue is more sensitive to oxidation. The order of rate of oxidation of polytetrapeptides is found to be poly(GGIP) > poly (GGVP) > poly(GGAP), which is in good agreement with their hydrophobicities.

Spectral evidence for the formation of PTP–Mn(III) complex

The study of the UV–visible spectra of pure Mn(III), PTP and a mixture of Mn(III) and PTP shows less deviation in peak wavelength (λ_{max}) and absorbance, as shown in Table 4.

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