EPR Spectroscopic Studies of the Reduction of Chromium(VI) by Methanol in the Presence of Peptides. Formation of Long-Lived Chromium(V) Peptide Complexes

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The synthesis and characterization of the first Cr(V) complexes with non-sulfur-containing peptides, which may mimic the chemistry of the intermediates in the formation of Cr-induced peptide−DNA cross-links in vivo, are reported. The reduction of Cr(VI) with methanol in the presence of a number of non-sulfur-containing peptides produced relatively stable Cr(V)−peptide complexes, which were characterized by EPR spectroscopy and electrospray mass spectrometry. The reaction of Cr(VI) with methanol alone (in the absence of peptide ligands) resulted in the formation of two Cr(V)−methanol intermediates, with g_{iso} values of 1.9765 and 1.9687. The methanol reduction of Cr(VI) in the presence of the glycine peptides, triglycine, tetraglycine, and pentaglycine resulted in the formation of both Cr(V)−methanol and Cr(V)−peptide intermediates, while only the Cr(V)−peptide complexes were detected in the reactions with the alanine peptides trialanine, tetraalanine, and pentaalanine. Similar EPR signals were observed for all of the Cr(V)−peptide complexes with g_{iso} values between ∼1.986 and ∼1.979, and A_N values of $(2.1-2.6) \times 10^{-4}$ cm^{-1}.

Introduction

Chromium(VI) is a known carcinogen; however, Cr(VI) alone does not damage DNA in vitro.[1] In the presence of reducing agents, such as ascorbate, glutathione, and cysteine, chromate is capable of causing a wide range of DNA lesions, which have been observed in both in vivo and in vitro studies.[2] It is generally believed that lower valent Cr intermediate species, i.e., Cr(V) and/or Cr(IV), or radical species are the active carcinogenic agents.[3] Ascorbate, which readily reduces Cr(VI) at pH 7.0, is reported to produce both Cr(V) and Cr(IV) intermediates as well as the ascorbate and carbon-based radicals.[4] Recent EPR studies of the reduction of Cr(VI) with ascorbate showed that the formation of Cr(V) species was dependent on the pH value and the concentration of ascorbate.[5] When ascorbate was reacted with excess Cr(VI), the formation of Cr(III) and the ascorbate radical was favored, while lower concentrations (e.g., Cr(VI):Asc = 2:1) favored the production of Cr(V)−ascorbate complexes, of which at least seven were EPR active.[5] The reactivity of intermediates formed during the reduction of Cr(VI) with ascorbate toward DNA in vitro showed that both binding and strand breaks occurred, and the damage was postulated to be due to Cr(V) and/or carbon-based radicals.[6] While the exact mechanisms of such damage are not yet fully understood, the detoxifying effects of ascorbate have been demonstrated in mammalian-cell in vitro studies.[7] Increasing the level of ascorbate in cells correlated directly with decreased Cr(VI) genotoxicities.[7]

Evidence supporting Cr(V) intermediates as the active agents in Cr(VI)-induced carcinogenesis has been demonstrated with Cr(V) model complexes. The relatively stable Cr(V) complex $[Cr^{V}O(ehba)_2]^-$, where ehba is a model for 2-hydroxy acid complexes, such as those of lactate and citrate, significantly cleaves DNA in vitro and is postulated to act via a ligand-exchange mechanism.[8,9] The stable Cr(V) complex formed with the peptide model ligand mampa,[10] $[Cr^{V}O(mampa)]^-$, is unable to undergo ligand-exchange reactions and exhibits only weak cleavage reactions with DNA. However, the complex is permeable and genotoxic to mammalian cells in vitro at levels similar to Cr(VI).[11] The generation of the Cr(V) peptide model complexes was achieved by oxidation of the Cr(III) analogue in buffer solutions at pH 3.8.[12] Similarly, Cr(V)−peptide complexes of triglycine, tetraglycine, and pentaglycine were easily generated in situ via oxidation of the Cr(III)−peptide complexes in buffer solutions at various pH values.[13] However, results from EPR and UV/vis studies indicated that these Cr(V) species are relatively unstable.

The potential importance of Cr complexes of peptides in Cr-induced cancers was indicated by the observation that Cr(VI) interacts with DNA in the presence of a microsomal enzyme

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fraction to produce DNA−protein cross-links.[14] Subsequently, it was shown that such cross-links are markers for human exposure to carcinogenic Cr(VI)[15] and that in cultured cells some of the cross-links involve non-sulfur-containing amino acid residues.[16] Since Cr(III) also causes the same cross-links,[16] the question arises as to which oxidation state is involved. Recently, it was shown that Cr(V)−peptide complexes could be generated intracellularly from Cr(III),[13] and Cr(V) tetraamide complexes damage DNA and are mutagenic in bacterial and mammalian cells.[11,17] Although the Cr(V) peptide complexes were generated from the oxidation of Cr(III) peptide compounds under physiological conditions of temperature and pH, none of the complexes were isolated, because of further oxidation reactions.[13] Thus we explored the potential synthesis of these complexes by peptide trapping of Cr(V) intermediates generated during the reduction of carcinogenic Cr(VI). Here we report the first isolation and characterization of a Cr(V)−peptide complex that does not contain thiol residues.

Experimental Section

Reagents. Triglycine, tetraglycine, pentaglycine, L-trialanine, L-tetraalanine, L-pentaalanine, D-trialanine, L-alaala-D-ala, alaglygly, glyglyhis, and hisglygly (all Sigma, 99%) were used without further purification. Other reagents were $Na_2Cr_2O_7 \cdot 2H_2O$ (Merck, 98%), methanol (Biolab Scientific, AR grade), acetone (BDH, 99.5%), and cyclohexane (Ajax Chemicals, 99%).

CAUTION: *Cr(VI) is a human carcinogen,[1,18] and Cr(V) complexes are mutagenic and potential carcinogens.[8,11] Appropriate precautions should be taken to avoid inhalation and skin contact while handling.*

EPR Spectroscopy. Room temperature (∼20 °C) X-band EPR spectra were obtained from solutions contained in a quartz flat cell using a Bruker EMX EPR spectrometer operating at ∼9.6 GHz with a Bruker EMX 081 magnet and an ERO 41XG mircowave bridge. The spectra were calibrated using a Bruker EMX 035M NMR gaussmeter in conjunction with an EMX 048T mircowave bridge controller and an EMX 032T field controller. Operating parameters for X-band EPR were as follows: power, 200 mW; modulation frequency, 100 kHz; modulation amplitude, 2.07, 1.07, or 0.57 G; receiver gain, 1×10^5 or 1×10^4; conversion time, 20.48 ms; time constant, 5.12 ms; sweep time, 20.972 s; center field, 3480 or 3500 G; sweep width, 100 or 200 G; temperature, ∼20 °C; and number of scans, 5.

Room temperature (∼20 °C) Q-band spectra were recorded using a Bruker ESP 300 spectrometer operating at ∼34 GHz with a Bruker ER 085C lMagnet and ER 041MR microwave bridge. Operating parameters for Q-band EPR were as follows: modulation frequency, 100 kHz; modulation amplitude, 0.9509 G; receiver gain, 5×10^3; conversion time 40.960 ms; time constant, 20.480 ms; sweep time, 41.943 s; center field, 12200 G; sweep width, 300 G; temperature, ∼20 °C; and number of scans, 10. Spectra were calibrated using standard DPPH (α,α'-diphenol-β-picrylhydrazol), which has a g_{iso} value of 2.0036.[19]

For spectra having overlapping signals from several different Cr(V) complexes, the WinSim EPR simulation program[20] was used to estimate the spectral parameters. Initial estimates of the peak positions, line widths, and their relative areas were obtained from the second-derivative spectra. The simulations did not always take into account the contributions from minor signals that arise from ^{53}Cr-hyperfine coupling. In the analysis of all EPR spectra, second-order corrections have been applied in the calculation of A_{iso} values.

Other Physical Measurements. Electronic absorption (UV/vis) spectra were obtained using a Hewlett-Packard 8452A diode array spectrophotometer over the range 190−820 nm. Compound and reaction solutions were contained in a quartz cell with a 1 cm path length. Infrared spectra were recorded using a Bio Rad FTS-40 spectrophotometer, and solid samples were prepared as ∼15% w/w KBr (Sigma, IR grade) mulls. Microanalyses were performed by the Australian National University Microanalytical Service. Electrospray mass spectra were obtained using a Finnigan LCQ ESI-APCI triple quadrupole mass spectrometer. Experimental conditions were as follows: spray voltage, 5 kV; nitrogen sheath gas pressure, 60 psi; heated capillary temperature, 200 °C; full scan, 50−2000 *m/z*. For loop injection, the mobil phase was 50% methanol/50% water with 1% acetic acid and the flow rate was 100 μL min^{-1}.

Reduction Reactions. In general, a MeOH solution of $Na_2Cr_2O_7 \cdot 2H_2O$ (50 mM) was prepared and added to a preweighed amount of peptide to give a solution of the ligand (200 mM) in a glass vial. The reaction was left for a given time in the presence of a fluorescent light (from a desk lamp, 15 W), and the EPR spectra were recorded every 3 days over a 2−3 week period, depending on the overall rate of the reaction. A control reaction was also prepared in the absence of peptide ligands, i.e., contained Cr(VI) and methanol alone, to determine if any of the Cr(V) EPR signals were due to Cr(V)−MeOH species. This reaction was performed in the presence and absence of light.

Cr(V)−Oligoalanine Complexes. The Cr(V) complexes of trialanine, tetraalanine, and pentaalanine were prepared by reacting $Na_2Cr_2O_7 \cdot 2H_2O$ (50 mM) and the appropriate peptide ligands in excess (400 mM) in methanol (∼2−5 mL). The reaction solutions were exposed to fluorescent light for 6−7 days at room temperature with constant stirring. After such time, the solutions had turned dark brown. The Cr(V)−trialanine complex was the only product to be purified and extensively characterized.

Cr(V)−L-Trialanine Complex. The Cr(V)−trialanine complex was prepared by reacting $Na_2Cr_2O_7 \cdot 2H_2O$ (0.12 g) and L-trialanine (0.5 g) in methanol (10 mL) in the presence of fluorescent light for 6 days with constant stirring of the reaction mixture at room temperature. Excess L-trialanine ligand and insoluble brown products were removed by filtration. The dark brown filtrate was treated with acetone (∼5 mL) to precipitate Cr(III) products, which were then removed by filtration. The filtrate was then treated with cyclohexane (∼5 mL) to remove any unreacted Cr(VI). The oily yellow-brown product was collected and dried in a desiccator for 48 h. Yield of dark brown product: 0.097 g (24%). Anal. Calcd for $Na[CrO(C_9H_{14}N_3O_4)(OCH_3)] \cdot 0.5NaOCH_3 \cdot 5H_2O$: C, 26.98; H, 6.15; N, 8.99. Found: C, 27.17; H, 5.35; N, 8.40. ES/MS (water/methanol): (+ve ion) 276 (27%), 358 (23%), 529 (100%) and 541 (44%) *m/z*; (-ve ion) 230 (16%), 327 (100%) and 426 (33%) *m/z*. UV/vis (water): 372 nm (3080 M^{-1} cm^{-1}). IR (KBr mull): 3305 (m, br), 2979 (w), 3935 (w), 1653 (s), 1559 (m), 1507 (w), 1457 (w), 1373 (w), 1245 (w), 1162 (w), 1060 (m), 908 (w), 779 (w), 668 (w), 526 (w), 473 (m), 458 (w), and 419 (m) cm^{-1}.

Results

Cr(VI)/Methanol Reaction. As a prelude to studying the Cr(V)−peptide complexes generated by the reduction of Cr(VI) in methanol, the reaction in the absence of the ligand was investigated. The control reaction changed color from an orange to brown within a few days after being exposed to light. If kept in the dark, the reaction solution remained orange for months, although very weak Cr(V) signals could be detected after several weeks. Similar observations were made for Cr(V) intermediates formed in the presence of peptide ligands, and thus all reaction solutions were exposed to light. The EPR spectrum of the control reaction had a maximum intensity after 3 days in the presence of fluorescent light (15 W lamp), and two Cr(V) signals were present with g_{iso} values of 1.9765 and 1.9687 (Figure 1). After day 18, a fine green precipitate began to form and the EPR

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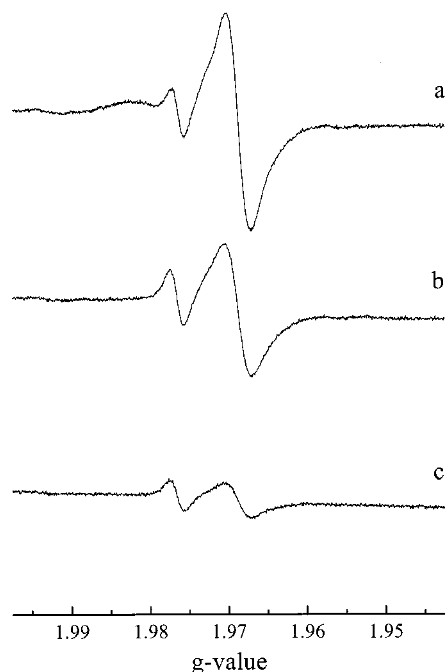


Figure 1. X-band EPR spectra of the Cr(V) intermediate complexes formed during the reduction of $Na_2Cr_2O_7{\cdot}H_2O$ (50 mM) in methanol after (a) 3, (b) 12, and (c) 18 days in the presence of light at ~20 °C. Center field = 3500 G, and sweep width = 100 G.

signal intensity decreased substantially. Subsequently, the intensity of the color faded and the solution was colorless at the end of 4 weeks. For the reduction of Cr(VI) in methanol in the presence of various peptides, the same color change from orange to brown was observed; however, the Cr(V) signals observed in the EPR spectra were different than those observed in the reaction containing methanol alone.

Glycine Peptides. The reduction of Cr(VI) in the presence of the glycine peptides triglycine, tetraglycine, and pentaglycine in the light produced a number of different Cr(V) signals. Exposure of Cr(VI) and triglycine to light for 3 days resulted in four Cr(V) signals with g_{iso} values of 1.9824, 1.9782, 1.9752, and 1.9688 (Figure 2a). After day 12, the intensities of the Cr(V) signals diminished, although the signal at 1.9824 become more prominent with respect to the other signals and five ^{14}N-superhyperfine coupling lines were observed ($A_N = 2.42 \times 10^{-4}$ cm^{-1}), showing coupling with two equivalent nitrogen atoms (Figure 2b). The reduction of Cr(VI) in the presence of tetraglycine also produced several Cr(V) EPR signals, some of which were overlapping (Figure S1a). The maximum intensity of the EPR signals was obtained at day 3 of the reaction, and at least five Cr(V) signals were observed with g_{iso} values of 1.9848, 1.9808, 1.9792, 1.9756, and 1.9689. After day 12 of the reaction, the intensities of the Cr(V) signals diminished somewhat and those at lower g_{iso} values (due to Cr(V)–methanol intermediates) became less prominent (Figure S1b). In addition, the ^{14}N-superhyperfine coupling structure of the overlapping Cr(V) signals at higher g_{iso} values was better resolved, and an A_N value of 2.28×10^{-4} cm^{-1} was calculated.

The reaction of Cr(VI) in methanol with pentaglycine in the presence of light also produced several Cr(V) species, some of which had g_{iso} values similar to those observed in the triglycine and tetraglycine reactions. Again the maximum signal intensity occurred after 3 days, and there were at least five Cr(V) signals with g_{iso} values of 1.9852, 1.9806, 1.9792, 1.9758, and 1.9689 (Figure S2). In addition, weaker signals at 1.9965 and 1.9635 were observed and are most probably due to ^{53}Cr hyperfine

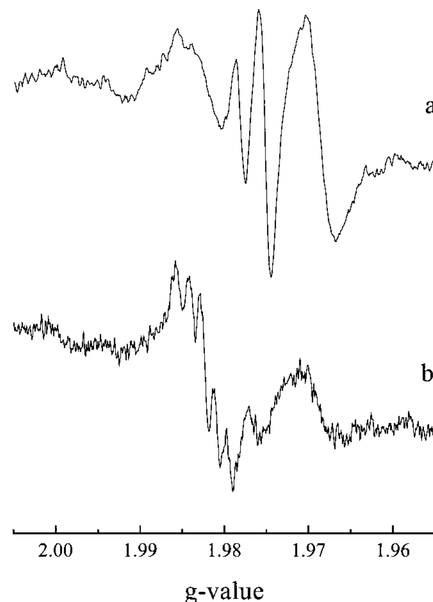


Figure 2. X-band EPR spectra of the Cr(V) intermediates formed during the reduction of $Na_2Cr_2O_7{\cdot}H_2O$ (50 mM) in methanol with triglycine (200 mM) after (a) 3 and (b) 12 days in the presence of light at ~20 °C, where the intensity of spectrum b is 1/167th the intensity of a. Center field = 3500 G, and sweep width = 100 G.

Table 1. EPR Spectroscopic Data for the Cr(V) Species Generated in the Reduction of Cr(VI) in Methanol with Oligoglycine Peptides at Room Temperature in the Presence of Light

reaction solution	g_{iso} value[a] (A_N value × 10^{-4} cm^{-1})				
MeOH control	1.9765				1.9687
triglycine	1.9824 (2.42)	1.9782		1.9752	1.9688
tetraglycine	1.9848 (2.28)	1.9808[b]	1.9792	1.9756	1.9689
pentaglycine	1.9852	1.9806[b]	1.9792	1.9757	1.9689

[a] All g_{iso} values were obtained from the second derivative spectra. [b] Signal may be due to several overlapping signals, similar to results obtained for oligoalanine peptides.

coupling. For the triglycine and tetraglycine complexes, hyperfine coupling due to ^{53}Cr was not observed over the noise due to the weaker signal intensities. All g_{iso} values for the reactions of Cr(VI) with oligoglycine peptides are summarized in Table 1. Spectra for the Cr(V) oligoglycine peptides were not simulated due to the complexity of the overlapping species but are similar to those observed in the oxidation of Cr(III)–polyglycine complexes.[13]

Alanine Peptides. The reduction of Cr(VI) by methanol in the presence of the peptides L-trialanine, L-tetraalanine, and L-pentaalanine exposed to light produced several Cr(V) complexes. The reaction with L-trialanine produced one Cr(V) signal, with a g_{iso} value of 1.9827, which possessed five-line ^{14}N-superhyperfine coupling ($A_N = 2.17 \times 10^{-4}$ cm^{-1}, Figure 3). By increasing the receiver gain parameter, the satellite signals due to ^{53}Cr-hyperfine coupling were resolved ($A_{Cr} = 17.79 \times 10^{-4}$ cm^{-1}), and each of these signals also possessed five-line ^{14}N-superhyperfine coupling structure. While the five-line coupling shows coordination of 2 equiv peptide nitrogen atoms, the simulation of the spectrum was achieved including contributions from a third nitrogen, i.e., the terminal amine group, with a much smaller coupling constant (0.42×10^{-4} cm^{-1}). The correspondence between the observed (Figure 3a) and the simulated (Figure 3b) was excellent (correlation coefficient = 0.996) due to the inclusion of ^{53}Cr-hyperfine coupling in the simulation and shows that only one species was present. In addition, the Q-band spectra also showed only one Cr(V) signal

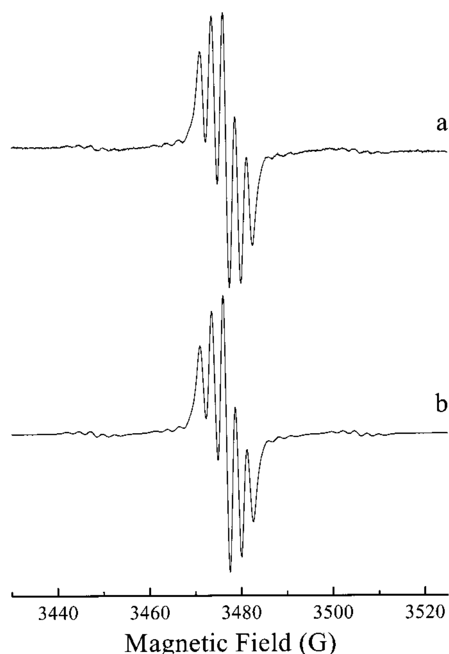


Figure 3. X-band EPR spectra of the Cr(V) intermediate complex formed during the reduction of $Na_2Cr_2O_7 \cdot H_2O$ (50 mM) in methanol with L-trialanine (200 mM) in the presence of light at ~20 °C after 6 days: (a) observed and (b) simulated; $\nu \approx 9.6581$ GHz. Spectrum b was simulated assuming one signal at 1.9827 (90.45%; 2 N, $a_{iso} = 2.31 \times 10^{-4}$ cm^{-1}; 1 N, $a_{iso} = 0.45 \times 10^{-4}$ cm^{-1}) and a second due to ^{53}Cr hyperfine (9.55%; 1 Cr, $A_{iso} = 17.88 \times 10^{-4}$ cm^{-1}; 2 N, $a_{iso} = 2.31 \times 10^{-1}$ cm^{-1}; 1 N, $a_{iso} = 0.45 \times 10^{-4}$ cm^{-1}) with a correlation coefficient = 0.996.

(not shown). None of the Cr(V) signals at lower g_{iso} values were observed, as was seen with the Cr(V)−triglycine reaction solutions.

The X-band EPR spectra of the reactions of Cr(VI) with L-tetraalanine and L-pentaalanine produced similar Cr(V)−peptide signals with at least two overlapping species (Figure 4a,b shows the reaction solutions at day 7). Similar to the Cr(V)−trialanine reaction, there were no Cr(V)−methanol intermediate signals observed. The overlapping signals were resolved by Q-band EPR spectroscopy (Figures 5 and S2). The results showed that the signals observed at X-band frequency split into two signals at Q-band frequency. A signal at $g_{iso} = 1.9851$ with resolved ^{14}N-superhyperfine structure was observed for both tetra- and pentaalanine reaction solutions. The second derivative spectra (not given) revealed at least seven coupling lines in a symmetrical arrangement, showing that the Cr(V) center was coordinated to three equivalent nitrogen atoms. The ^{14}N-superhyperfine structure of the other signal (at lower g_{iso} values) was not resolvable. Changing the modulation amplitude or receiver gain parameters did not enhance ^{14}N-superhyperfine structure and only resulted in noisy spectra. However, the second derivative spectra revealed that there were at least seven coupling lines, but these were in an unsymmetrical arrangement. It must also be noted that the intensity of signals at lower g_{iso} values was greater for shorter reaction periods, i.e., for 3−6 days. As the reduction reaction proceeded, the signals at higher g_{iso} values became more prevalent, i.e., for 7−12 days.

Simulation of the Q-band spectra of the Cr(V)−L-tetraalanine complexes was achieved only by assuming that more than two signals were present. The best fit occurred when five signals were used in the simulation of the spectra, and the correspondence between the observed (Figure 5a) and the simulated (Figure 5b) was quiet good (correlation coefficient = 0.979).

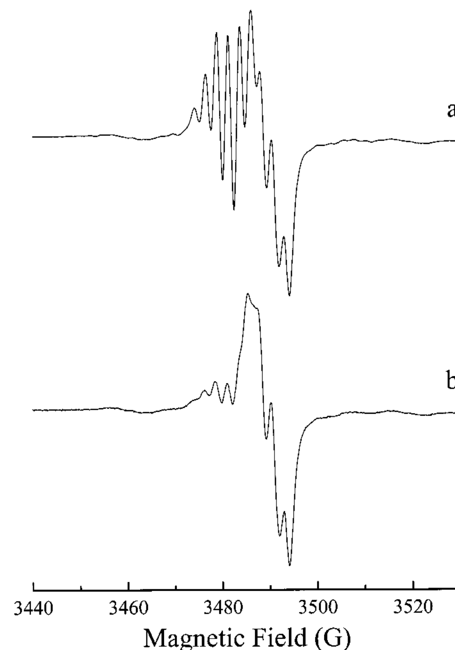


Figure 4. X-band EPR spectra of the Cr(V) intermediate complexes formed during the reduction of $Na_2Cr_2O_7 \cdot H_2O$ (50 mM) in methanol with (a) L-tetraalanine (200 mM) and (b) L-pentaalanine (200 mM) both in the presence of light at ~20 °C after 7 days.

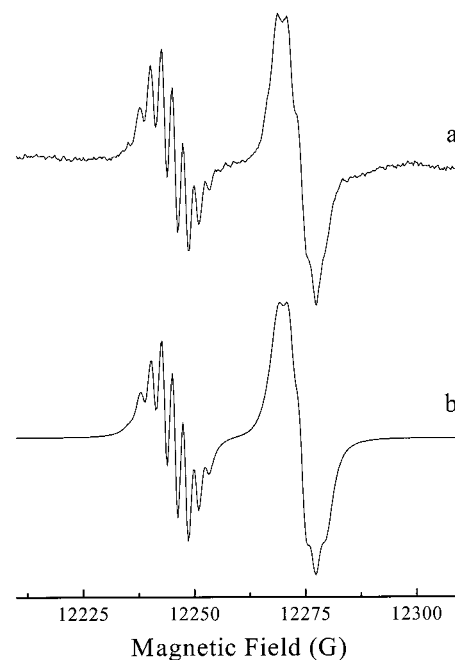


Figure 5. Q-band EPR spectra of the Cr(V) intermediate complexes formed during the reduction of $Na_2Cr_2O_7 \cdot H_2O$ (50 mM) in methanol with L-tetraalanine (200 mM) in the presence of light at ~20 °C after 7 days: (a) observed and (b) simulated; $\nu \approx 34.01$ GHz. Spectrum b was simulated assuming five signals at 1.9855 (6.22%; 3 N, $a_{iso} = 2.18 \times 10^{-4}$ cm^{-1}), 1.9851 (30.68%; 3 N, $a_{iso} = 2.18 \times 10^{-4}$ cm^{-1}), 1.9806 (19.16%), 1.9805 (12.39%; 2 N, $a_{iso} = 2.17 \times 10^{-4}$ cm^{-1}), and 1.9804 (31.55%; 2 N, $a_{iso} = 2.17 \times 10^{-4}$ cm^{-1}); correlation coefficient = 0.979. Average of last three g_{iso} values = 1.9805.

There were two overlapping signals at $g_{iso} = 1.9855$ and 1.9851, due to two Cr(V) species, both possessing seven-line ^{14}N-superhyperfine structure due to coupling with three equivalent nitrogens. The remaining three signals were overlapping with g_{iso} values of 1.9806, 1.9805, and 1.9804. The latter two signals also had ^{14}N-superhyperfine structure due to coupling with two

Table 2. EPR Spectroscopic Data for the Reduction of Cr(VI) in Methanol with Various Tripeptides at Room Temperature in the Presence of Light

peptide	$g_{iso\ value}$	$A_{iso\ value}$ ($\times 10^{-4}$ cm^{-1})	
L-trialanine[a]	1.9827	$A_N = 2.18$	$A_{Cr} = 17.77$
D-trialanine[b]	1.9826	$A_N = 2.25$	$A_{Cr} = 17.80$
L-alaala-D-ala[b]	1.9825	$A_N = 2.28$	$A_{Cr} = 17.81$
alaglygly[b]	1.9822	$A_N = 2.36$	$A_{Cr} = 17.73$
hisglygly[a]	1.9852	$A_N = 2.63$	
glyglyhis[a]	1.9847	$A_N = 2.34$	
	1.9791		
	1.9773		

[a] The g_{iso} and A_{iso} values were obtained from the simulated spectra. [b] The g_{iso} and A_{iso} values were obtained from second derivative spectra.

Table 3. Q-Band EPR Spectroscopic Data Calculated from Simulation of Spectra Obtained from the Reduction of Cr(VI) in Methanol with Oligoalanine Peptides at Room Temperature in the Presence of Light

peptide	$g_{iso\ value}$	A_N value[a]	N-mult	area (%)
L-tetraalanine	1.9855	2.18	3	6.22
	1.9851	2.18	3	30.68
	1.9806			19.16
	1.9805	2.17	2	12.39
	1.9804	2.17	2	31.55
L-pentaalanine	1.9855	2.18	3	3.92
	1.9851	2.18	3	22.68
	1.9808	2.17	1	8.58
	1.9805	2.17	1	55.00
	1.9796	2.17	2	9.82

[a] 10^{-4} cm^{-1}.

equivalent nitrogen atoms (Table 3). The X-band spectrum was then simulated using the parameters obtained from the Q-band simulation. However, while a good fit was obtained for the signals at higher g_{iso} values, i.e., at 1.9855 and 1.9851, the signals at lower g_{iso} values did not fit the spectra. As yet, a unique simulation has not been found that satisfied both the Q- and X-band spectral simulation.

Similar results were obtained for the Cr(V)−L-pentaalanine reaction solutions, and simulation of the Q-band spectra was again achieved assuming five signals. The correspondence between the observed (Figure S2a) and the simulated (Figure S2b) was good (correlation coefficient = 0.945). Similar to the Cr(V)−L-tetraalanine spectrum, there were two signals at g_{iso} = 1.9855 and 1.9851, both with seven-line ^{14}N-superhyperfine structure due to three equivalent nitrogen atoms, and three overlapping species with g_{iso} values of 1.9807, 1.9804, and 1.9798. The latter three signals were simulated with contributions from one, one, and two nitrogen atoms coordinated to the Cr(V) center, respectively. Similar to the Cr(V)−L-tetraalanine simulations, the X-band spectrum was then simulated using the parameters obtained from the Q-band simulation. However, again a unique simulation was not found that satisfied both frequency bands. A summary of EPR spectroscopic data obtained from the simulation of Q-band spectra is given in Table 3.

Other Tripeptides. The EPR spectra of the reduction of Cr(VI) in methanol with several other tripeptides were also recorded. For reactions involving D-trialanine, L-alaala-D-ala, and alaglygly, EPR signals similar to those observed for the L-trialanine complexes were apparent (see Table 2 for g_{iso} values). The EPR signals all possessed five-line ^{14}N-superhyperfine coupling involving two equivalent nitrogen atoms. The reduction reaction with hisglygly produced a Cr(V) signal with a g_{iso} value of 1.9852 (Figure 6a). The ^{14}N-superhyperfine

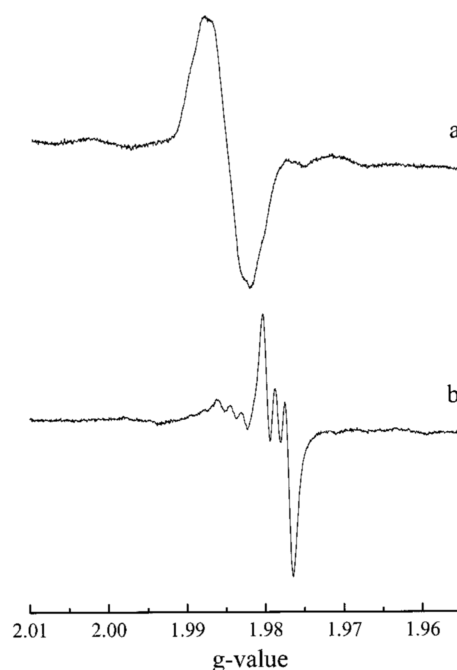


Figure 6. X-band EPR spectra of the Cr(V) intermediate complexes formed during the reduction of $Na_2Cr_2O_7 \cdot H_2O$ (50 mM) in methanol with (a) hisglygly (200 mM) and (b) glyglyhis (200 mM) at ~20 °C after 1 day. Center field = 3500 G, and sweep width = 100 G.

structure was not well resolved, and decreasing the modulation amplitude (to 0.57 G) or receiver gain (to 2×10^4) parameters did not fully resolve the coupling. However, the second derivative spectrum was sufficiently resolved to enable an A_N value to be determined as 2.63×10^{-4} cm^{-1}. The broadness of the signal suggests coupling with at least three nitrogen atoms. The spectrum was simulated assuming one main signal at 1.9853, with coupling to three nitrogen atoms, and four other minor signals. The correspondence between the observed and simulated spectra (not given) was somewhat lower than previous systems, with a correlation coefficient of only 0.984. The reaction of Cr(VI) with glyglyhis produced several overlapping signals with g_{iso} values of 1.9848, 1.9791, and 1.9772 (Figure 6b), and ^{14}N-superhyperfine coupling lines were observed (A_N = 2.34×10^{-4} cm^{-1}). The correspondence between the observed and simulated spectra (not given) was again lower than the previous systems (correlation coefficient = 0.976).

Isolation and Properties of Cr(V)−Oligoalanine Complexes. The reduction of Cr(VI) in methanol with L-trialanine was the only reaction to yield a single Cr(V) signal in the EPR spectrum, and this signal had a maximum intensity after 6 days. Elemental analysis of the solid product was consistent with the formula $Na[CrO(alaalaala)(OCH_3)] \cdot 0.5NaOCH_3 \cdot 5H_2O$. The UV/vis spectrum of the isolated complex was also consistent with the presence of Cr(V) and had a λ_{max} of 372 nm, which is similar to Cr(V)−peptide complexes formed via the oxidation of Cr(III) analogues.[21] The negative-ion ES/MS for the complex gave a main peak at 327 m/z (with 100% abundance), which can be assigned to the complex $[CrO(alaalaala)(OCH_3)]^-$. When the Cr(V)−L-trialanine complex was dissolved in water, the aqueous solution EPR spectrum gave the same five-line ^{14}N-superhyperfine coupled signal that was observed in the methanol reaction mixture. The correspondence between the observed spectra (Figure 7a) and the simulated spectra (not shown) was excellent, with a correlation coefficient of 0.994. The Cr(V)−

(21) Krumpolc, M.; Roček, J. *J. Am. Chem. Soc.* **1979**, *101*, 3206−3209.

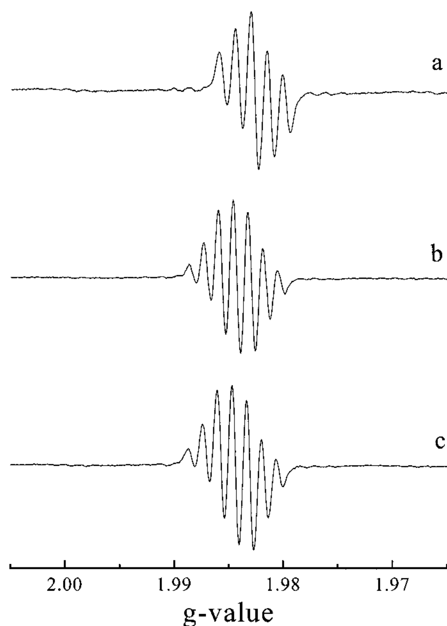


Figure 7. X-band EPR spectra of Cr(V) complexes of (a) trialanine, (b) tetraalanine, and (c) pentaalanine in water. Center field = 3490 G, and sweep width = 100 G.

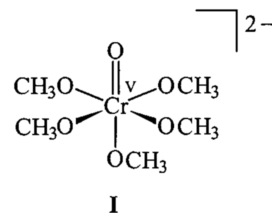
L-trialanine signal had g_{iso}, A_{Cr} and A_N values of 1.9828, 17.77 × 10^{-4} cm^{-1}, and 2.18 × 10^{-4} cm^{-1}, respectively.

The Cr(V) complexes of L-tetraalanine and L-pentaalanine were isolated as impure compounds, with contaminations from Cr(III) and Cr(VI). However, when the complexes were dissolved in water, the aqueous solution EPR spectrum revealed the same Cr(V) signal. Simulation of both spectra gave excellent correspondence between the observed (Figure 7b,a) and the simulated spectra (not shown), with correlation coefficients of 0.996 and 0.994 for the L-tetraalanine and L-pentaalanine complexes, respectively. For both complexes, there was a single signal at g_{iso} = 1.9844 with seven-line ^{14}N-superhyperfine coupling (A_N = 2.17 × 10^{-4} cm^{-1}) due to coordination of three equivalent nitrogen atoms. The second derivative spectra also showed seven coupling lines in a symmetrical pattern (not given), compared with the EPR spectra obtained for the reaction solutions, which gave at least nine-coupling lines with an irregular pattern due to there being several overlapping Cr(V) signals. This simplification of the spectra, along with the shift in g_{iso} value, indicates that a different Cr(V) complex forms in aqueous solution. For the Cr(V)−L-pentaalanine complex, the pH value of the aqueous solution was 8.6. In phosphate buffers, at pH values of 7.4 and 9.2, there was no change in the EPR spectrum. Similarly, no changes were observed in the EPR spectra of the Cr(V)−L-tetraalanine complex in phosphate buffers (pH 7.4 and 9.2) compared with the aqueous solution spectra (pH 8.2). However, in acetate buffer at pH 4.1, the intensity of the seven-line EPR signals decreased somewhat and a weaker narrower signal at ∼1.978 was observed.

Discussion

Initially, attempts to prepare Cr(V) complexes of small peptides via the reduction of Cr(VI) using the method of Krumpolc and Roček[21] were unsuccessful. The method required stirring a solution containing Cr(VI) and the ligand in acetone for ∼24 h in the dark, but the method failed for the peptide ligands presumably due to low solubility and weakly reducing properties of the peptides. Therefore, it was necessary to overcome two problems in the synthesis of the Cr(V) peptide complexes. The first was to find a suitable solvent in which the starting materials were soluble, and the second, since the non-sulfur-containing ligands did not reduce Cr(VI) themselves to any appreciable extent, was to use a solvent capable of reducing Cr(VI) to Cr(V). The most successful solvent used to date in the syntheses of Cr(V) peptide and amino acid complexes was methanol, which was used previously in the synthesis of the Cr(V) quinic acid complex.[22] In these reactions, dichromate acts as both the Cr(VI) reactant and the source of base to deprotonate the peptide ligands.[23] Although the peptides were sparingly soluble in methanol, the methanol reduced Cr(VI) to Cr(V)−OMe complexes, which were sufficiently stable to enable coordination of the peptide ligands by ligand-exchange reactions. Reactions performed in water yielded no EPR signals, and it can be presumed that the ligands play little or no part in the reduction of Cr(VI). All reactions required light to catalyze the reduction of Cr(VI) to the Cr(V)−OMe intermediates at a reasonable rate. It is not clear whether light also catalyzed the Cr(V) ligand-exchange with the peptide ligands, but this is not a requirement for coordination because the alanine peptide complexes also formed in the dark.

Characterization of the Cr(V)−Methanol Intermediate Species. The reaction of Cr(VI) in methanol alone yielded two EPR active signals at 1.9765 and 1.9687. The former signal is consistent with a six-coordinate Cr(V)−oxo species, **I**, and has a calculated g_{iso} value of 1.9759 based on the isotropic EPR parameters for oxygen donor ligands.[24] The latter signal, at g_{iso} = 1.9687, is most probably a mixed-valent dinuclear Cr(V)−Cr(VI) or Cr(V)−Cr(III) species, by analogy with Cr(V)−oxalate systems.[25] However, further work would be required to determine the exact nature of this species.



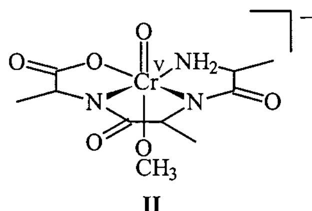
Characterization of the Cr(V)−L-Trialanine Complex. The strong EPR signal of the Cr(V)−L-trialanine complex in water is most likely due to the same complex that was observed in the ES/MS. Therefore, it was deduced that the observed signal at g_{iso} = 1.9828 is due to the six-coordinate species in methanol, **II**, and an analogous OH^- complex in water, **III**. The five-line ^{14}N-superhyperfine structure is due to the coupling of two peptide nitrogen atoms, and the broadness of the signal compared to the other peptides suggests that ^{14}N-superhyperfine structure due to the amine nitrogen is much smaller and is not resolved in this signal. In the larger peptides, the amine group would not be bound, leading to sharper signals.

(22) Codd, R.; Lay, P. A. *J. Am. Chem. Soc.* **1999**, *121*, 7864−7876. Codd, R.; Levina, A.; Zhang, L.; Hambley, T. W.; Lay, P. A. *Inorg. Chem.* **2000**, *39*, 990−997.

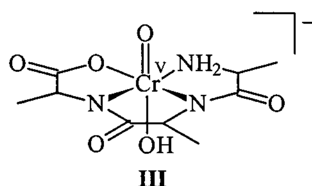
(23) The redox reaction is consistent with the stoichiometry below in which the dichromate acts as both the source of Cr(VI) and as the base. Although the reaction is clearly more complicated that indicated by the reaction below, it shows that no additional base is required to deprotonate the peptide during the coordination of the ligand. $Cr_2O_7^{2-} + 3CH_3OH + 2ala_3H_3 \rightarrow 2[CrO(ala_3)(OCH_3)]^- + H_2CO + 5H_2O$.

(24) Barr-David, G.; Charara, M.; Codd, R.; Farrell, R. P.; Irwin, J. A.; Lay, P. A.; Bramley, R.; Brumby, S.; Ji, J. Y.; Hanson, G. R. *J. Chem. Soc., Faraday Trans.* **1995**, *91*, 1207−1216.

(25) Farrell, R. P.; Lay, P. A.; Levina, A. Maxwell; I. A.; Bramley, R.; Brumby, S.; Ji, J.-Y. *Inorg. Chem.* **1998**, *37*, 3159−3166.



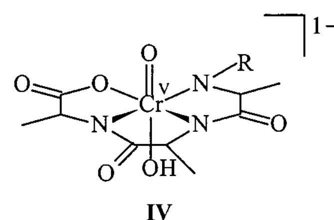
II



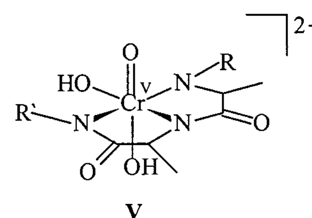
III

Isotropic EPR Parameters for Cr(V)−Peptide Complexes. The isotropic EPR parameters for Cr(V) complexes in solution with various oxygen donor atoms have been determined for both five- and six-coordinate species. Five-coordinate Cr(V) complexes have higher g_{iso} values and lower A_{iso} values than the corresponding six-coordinate Cr(V) complexes. In addition, Cr(V) complexes with mixed-donor ligands exhibit linear correlations between the number of donors of each type and the g_{iso} values. It is expected that Cr(V) complexes with nitrogen donor atoms would display similar correlations. There are very few examples of well-characterized Cr(V) complexes with nitrogen donor ligands. The macrocyclic tetraamide complexes $[Cr^VO(mac)]^-$ and $[Cr^VO(mampa)]^-$ are both five-coordinate,[10,12] and the EPR spectra of both complexes exhibit nine-line ^{14}N-superhyperfine coupled signals with g_{iso} values of 1.999 and 2.006, respectively (see Table S1 for A_{iso} values). The nine-line structure is due to coupling of the Cr(V) center with four equivalent amide nitrogen atoms. These complexes were extremely stable due to the macrocyclic nature of the ligands, and the signal of $[Cr^VO(mampa)]^-$ remained unchanged over a 4 h period at 37 °C.[17] The Cr(V) imine complex, $[Cr^VO(salen)]^+$,[10] is also five-coordinate and exhibits an EPR signal at $g_{iso} = 1.978$ with a five-line ^{14}N-superhyperfine structure due to coupling with two equivalent nitrogens[26] (Table S1). Recently, Cr(V) complexes with oligoglycine peptide ligands were prepared via the oxidation of the Cr(III) analogous complexes in various buffered solutions.[13] The most stable Cr(V) complexes were those with pentaglycine, which exhibited a seven-line ^{14}N-superhyperfine coupled signal at $g_{iso} = 1.9844$ in both acetate (pH 3.85) and phosphate (pH 7.4) buffers. The lower g_{iso} value of the complex, compared with the macrocyclic tetraamide complexes, shows that the Cr(V)−pentaglycine complexes cannot be five-coordinate with an oxo group and four deprotonated peptide amide groups (since a seven-line splitting was observed due to coupling with three peptide amide groups). A similar EPR signal was observed for the Cr(V)−tetraglycine complex at pH 7.4 (phosphate buffer). The assignments of the structures of the Cr(V)−peptide complexes is based on the six-coordinate Cr(V)−trialanine complex and isotropic empirical parameters of 1.9925 for the peptide amide group and 1.9825 for the amine group for six-coordinate species.

Characterization of Cr(V) Signals at $g_{iso} = 1.9844$. The aqueous solution EPR spectra of Cr(V) complexes of L-tetraalanine and L-pentaalanine (formed via reduction reactions) had signals similar to those observed with the Cr(V)−pentaglycine complex. Both complexes had Cr(V) signals with a g_{iso} value of 1.9844, which possessed seven-line ^{14}N-superhyperfine structure due to coordination of three amide nitrogen groups (Figure 7b,c). The Cr(V) signals observed here must be due to similar Cr(V) species and are consistent with structure **IV**, which has calculated g_{iso} values of 1.9844. The R group represents the terminal amine end of the peptide, where $R = COCH_2NH_3^+$ for L-tetraalanine and $COCH_2NCOCH_2NH_3^+$ for L-pentaalanine. Chromium(V) complexes with peptide ligands coordinated in a tridentate manner, as in structure **V**, have a similar calculated g_{iso} value of 1.9847, but are unlikely.

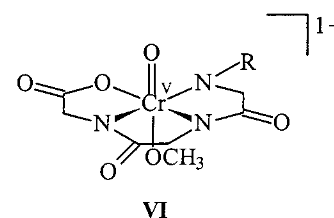


IV

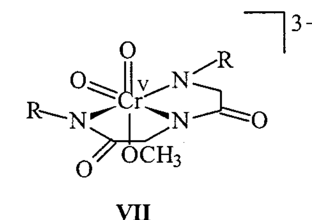


V

Characterization of Cr(V) Signals at $g_{iso} \approx 1.985$. The simulation of the Q-band EPR spectra of the methanol reaction solution of the tetra- and pentapeptides of alanine showed two signals at $g_{iso} = 1.9855$ and 1.9851, both possessing seven-line splitting due to three coordinated amide nitrogen atoms. Similarly, X-band EPR spectra of tetraglycine and pentaglycine Cr(V) complexes had signals at 1.9848 and 1.9852, respectively. The most likely assignment of these signals are a methanol analogue of the signal observed at 1.9844, such as structure **VI**, which has a calculated g_{iso} value of 1.9850.



VI



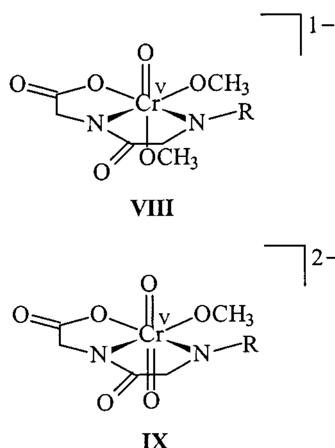
VII

A less likely assignment for the complex that gives rise to the signal is structure **VII**, in which the peptide ligand is coordinated in a tridentate manner, and the fourth and fifth positions are occupied by methoxyl and oxo groups, giving a calculated g_{iso} value of 1.9855.

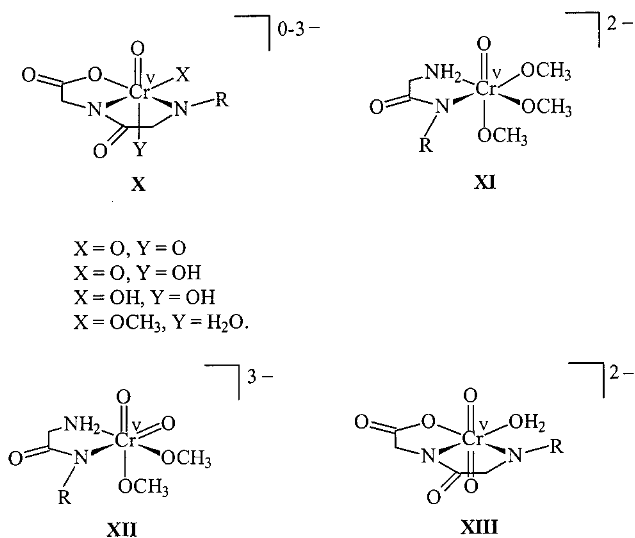
Characterization of Other Cr(V) Signals. The Q- and X-band EPR spectra of methanol reaction solutions of L-tetraalanine and L-pentaalanine showed that several overlapping signals were present at lower g_{iso} values. These signals were more prominent at the beginning for the reduction reaction and

(26) Srinivasan, K.; Kochi, J. K. *Inorg. Chem.* **1985**, *24*, 4671−4679.

decreased in intensity as the reaction progressed. Simulation of both X- and Q-band spectra revealed that at least three signals with one- or two-coordinated nitrogens were present. Therefore, these signals are probably due to Cr(V)−peptide intermediate species with the peptide ligands coordinated in a bi- or tridentate manner, with methoxyl, hydroxy, aqua, and oxo groups in the remaining positions. This is consistent with their appearance in the early stages of the reaction, with the denticity of the peptide of the predominant complexes increasing as the reaction proceeds. Possible assignments for the signal at g_{iso} 1.9813 with two coordinated nitrogen atoms are structures **VIII** and **IX**, which have calculated g_{iso} values of 1.9816 and 1.9813, respectively.

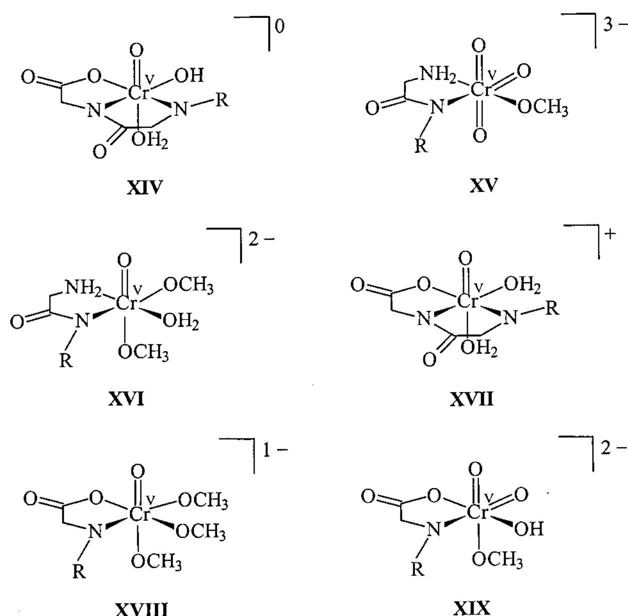


There are several possible assignments for signals observed between 1.9808 and 1.9804, and structure **X** outlines complexes with two coordinated nitrogens. As the ligands at X and Y are changed, the calculated g_{iso} values are 1.9809, 1.9807, 1.9805, and 1.9805, respectively. The assignment of the complex with only one peptide nitrogen is structure **XI**, which has a calculated g_{iso} value of 1.9805. Structures such as **XII** and **XIII** have calculated g_{iso} values of 1.9802 and 1.9801, respectively.



Signals at lower g_{iso} values of 1.9798 and 1.9792 could also have several different assignments; some examples are outlined below in structures **XIV**−**XVII**. The calculated g_{iso} values for the structures are 1.9799, 1.9798, 1.9794, and 1.9793.

The Cr(V) signal at 1.9782, which was observed in the reduction of Cr(VI) with triglycine, is assigned to a Cr(V)−methanol−peptide intermediate, as in **XVIII** (calculated g_{iso} value = 1.9783). The signal at 1.9773, which was observed in the reduction reaction of Cr(VI) with glyglyhis is most likely due to a similar complex, as in structure **XIX** (calculated g_{iso} value = 1.9774). The remaining EPR signal observed between



1.9752 and 1.9756 and 1.9687−1.9689 in the oligoglycine spectra are assigned to Cr(V)−methanol intermediates, as discussed earlier.

Biological Implications of Cr(V)−Peptide Complexes. The potential role of Cr(V) complexes in Cr(VI)-induced genotoxicities has been demonstrated with the non-sulfur-containing peptide model complexes $[Cr^{V}O(mampa)]^-$ and $[Cr^{V}O(mac)]^-$, which were produced by the oxidation of the Cr(III) analogue complexes. Both complexes, while only cleaving DNA weakly in vitro, were permeable and genotoxic in Chinese hamster V79 lung cells.[17] The oxidation of Cr(III)−peptide complexes of triglycine, tetraglycine, and pentaglycine showed that several Cr(V) complexes were formed and are dependent on the oxidation time and pH value of the solution.[13] At low pH values (3.85), the oxidation reactions favored production of Cr(V) species and resulted in degradation to Cr(III), while oxidation reactions at higher pH values resulted in polymeric species and Cr(VI) end products.[13] Thus, such Cr(V) species could be generated in vivo from Cr(III) peptides. Here, we have shown that quite stable Cr(V)−peptide complexes can also be formed via the reduction of Cr(VI) in methanol. Attempts at isolating pure Cr(V)−peptide complexes from the oxidation of Cr(III) analogues resulted in mixtures of Cr(III), Cr(V), and Cr(VI).[13] Similarly, isolation of Cr(V) peptide complexes from the reduction of Cr(VI) also produced mixtures of Cr(III), Cr(V), and Cr(VI). While, the Cr(III) content could be removed, due to its insolubility in aqueous solution, the Cr(V) complex has not, as yet, been separated from Cr(VI), except in the case of the trialanine complex. It may well be that the Cr(V) complexes are undergoing disproportionation to Cr(III) and Cr(VI) or that the Cr(VI) content is due to starting material. If so, it is most likely that while Cr(VI) is readily reduced to relatively stable Cr(V) species, these then rapidly undergo further reduction to form insoluble Cr(III) complexes. While the biological activity of these complexes is lower than that of Cr(VI) and Cr(V)−peptide model complexes,[27] further work is required to obtain

(27) Headlam, H. A. PhD Thesis, University of Sydney, 1999.

a range of pure Cr(V)−peptide complexes and eliminate contributions of Cr(VI) to their biological activities.

While most of these Cr(V)−peptide complexes are yet to be obtained pure, they are genotoxic in the bacterial and micronucleus assays and bind to DNA in vitro,[27] which mimics the behavior of both Cr(VI) and Cr(III) DNA damage in cells.[15,16] Such damage has been attributed to Cr(V)−peptide complexes[13] that can be produced from Cr(III) complexes that are oxidized by oxo-transfer oxidants that mimic the oxidants found in vivo or, as shown here, by the reduction of Cr(VI).

Conclusions. The Cr(V) complexes of oligoglycine, oligoalanine, and other tripeptides are produced from the reduction of Cr(VI) in methanol. EPR spectroscopic studies illustrated that several different Cr(V) species were formed; the natures of these species were dependent on the particular peptide and the reaction period.

Acknowledgment. This work was supported by the Australian Research Council (ARC RIEFP grants for EPR and ES/MS instrumentation), an ARC Large grant, and a Worksafe Australia Scholarship. The authors also thank Dr. Ming Xie for assistance with Q-band EPR and Dr. Xiaomin Song for collection of ES/MS.

Supporting Information Available: Table 1S gives EPR spectroscopic data for well-characterized Cr(V) complexes with some nitrogen donor ligands. Figures S1 and S2 show the X-band EPR spectra of the reduction of Cr(VI) in methanol in the presence of tetraglycine and pentaglycine, respectively. Figure S3 shows the Q-band EPR spectra of the reduction of Cr(VI) in methanol in the presence of pentaalanine and simulated spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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