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Formation of oxo-centered trinuclear chromium carboxylate complexes and hydrolysis of Cr3 as established by paramagnetic ^{2}H NMR spectroscopy

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ABSTRACT

Paramagnetic ^{2}H NMR techniques have been utilized to study the mechanism of formation of the oxo-bridged trinuclear Cr(III) carboxylate assembly $[Cr_3O(O_2CCD_3)_6(H_2O)_3]^+$ from $[Cr(H_2O)_6]^{3+}$ and d_4-acetic acid. These studies reveal a complex mechanism dominated by the involvement of dinuclear intermediates. The oxo-bridged trinuclear Cr(III) carboxylate assembly $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ has been suggested for use as a chromium nutritional supplement and therapeutic agent as it is readily absorbed and has been proposed to enter cells intact. The paramagnetic ^{2}H NMR technique has been utilized to follow the stability of this Cr(III) carboxylate assembly in biologically relevant media; its stability is consistent with the assembly being able to enter cells intact.

1. Introduction

For the last three decades interest in oxo-centered trinuclear Cr carboxylate assemblies has steadily increased (Fig. 1). Broadly applicable synthetic routes to both symmetric and unsymmetric assemblies are now available, which have resulted in a large number of complexes of this type being synthesized and well characterized [1].

The works by Vincent and coworkers have shown that NMR is an effective method for characterization of these assemblies [2–10]. Although the Cr(III) centers are paramagnetic with an expected magnetic moment of 3.87 B.M., antiferromagnetic coupling between the metals gives an average magnetic moment of circa 3.4 B.M. allowing for useful spectra to be obtained. This technique has been shown to be efficient in assigning the spectra of Cr carboxylate complexes of simple aliphatic carboxylates as well as aromatic carboxylates. Notably, these studies illustrated the utility of ^{2}H NMR in assigning these systems; the deuterium signal is sharper than the corresponding proton signal by up to 42.5 fold. This difference is based on the difference in the gyromagnetic ratios of the two isotopes, $\gamma_H^2/\gamma_D^2 = 42.5$ where γ_H and γ_D are the gyromagnetic ratios of proton and deuteron, respectively [11]. Therefore, using deuterated starting material allows for observation of some signals that are too broad to detect by ^{1}H NMR. The signals are, therefore, better resolved while the chemical shift, relative to the ^{1}H NMR spectra, remains unchanged.

Although much has been accomplished to increase the number of oxo-centered trinuclear chromium carboxylate complexes and to increase the methods for their characterization, the one piece of information that is still in question is the mechanism by which these assemblies are formed. When examining the structure of the oxo-centered Cr carboxylates (Fig. 1), many possible mechanisms seem feasible for their formation. For example, the simplest mechanism to imagine is for mononuclear Cr carboxylates to combine to form dinuclear oxo-bridged carboxylates, which then combine with a third monomer to form the trinuclear assembly.

A review of the literature shows that there has been some insight into this process. In 1990, Lippard and coworkers reported the isolation and characterization of a blue colored dinuclear complex $[Cr_2(\mu\text{-}OH)(\mu\text{-}O_2CH)_2(H_2O)_6]^{3+}$ [12]. Within this complex two chromium(III) ions are bridged by one hydroxide and two bidentate formato ligands; the octahedron of each chromium atom is completed by three terminal water ligands. Lippard and coworkers reported that if during the preparation of this dinuclear complex the temperature was allowed to exceed 50 °C a green material gradually formed. This green material was identified by visible spectroscopy as the trinuclear assembly $[Cr_3O(O_2CH)_6(H_2O)_3]^+$. This suggested that the dinuclear complex might be an intermediate in the formation of the trinuclear complex. To test this hypothesis, Lippard and coworkers followed the reaction of chromic chloride and formic acid by visible spectroscopy. The reaction was heated to 70 °C; during this time the reaction solution changed from the green of the chromic chloride to a blue color, indicative of the dinuclear complex, and finally to a dark green. The spectra collected during the blue phase of the reaction were consistent with the isolated dinuclear complex, while spectra collected after the

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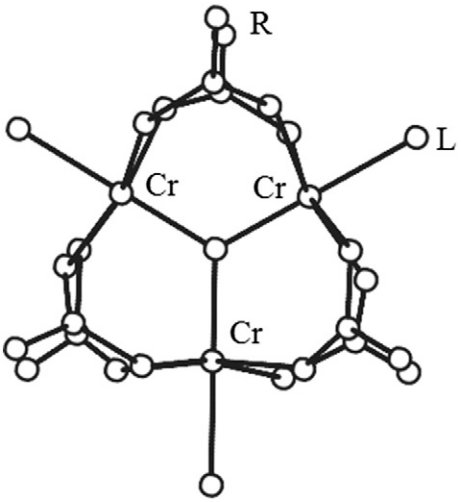

Fig. 1. Structure of the $[Cr_3O(O_2CR)_6]^+$ core. For Cr3, R = ethyl, and L = H_2O.

reaction returned to green were consistent with those of the trinuclear complex. The same procedure was carried out for the reaction of chromic chloride and acetic acid; however, the analogous dinuclear assembly could not be isolated. The conversion of the dinuclear complex into the trinuclear complex was reversed by the addition of water. These results strongly suggest that dinuclear complexes of the form $[Cr_2(\mu\text{-}OH)(\mu\text{-}O_2CR)_2(L)_6]^{3+}$ are intermediates in the formation of oxo-centered trinuclear complexes of the form $[Cr_3O(O_2CR)_6(L)_3]^+$.

In 1996, Spiccia and coworkers isolated a dinuclear complex of the form $[Cr_2(\mu\text{-}OH)(\mu\text{-}O_2CCH_3)(H_2O)_8]^{4+}$ from the reaction of "chromium(III) hydrolytic dimer" with acetic acid at pH = 4 [13]. Within this complex two Cr(III) ions are bridged by one hydroxide and one bidentate acetate; the octahedron of each chromium atom is completed by four terminal water ligands. Though the theory was not tested, Spiccia suggested this dinuclear assembly could also be involved in the formation of the trinuclear assemblies.

While these dinuclear complexes have been suggested as possible intermediates, to date no detailed study of the formation of oxo-centered trinuclear complexes has appeared. This is because the features in the visible spectra of the dinuclear and trinuclear complexes are very similar, broad and overlapping, not allowing for ready discernment of the species present in solution. Herein will be discussed the use of ^2H NMR to follow the reaction of Cr(III) nitrate with d_4-acetic acid in hopes of identifying the number and identity of the intermediates involved in the formation of the oxo-centered trinuclear Cr carboxylate $[Cr_3O(O_2CCH_3)_6(H_2O)_3]^+$.

The Vincent group, in preparing a series of anion-bridged Cr(III)–carboxylate complexes to generate a library of spectra for comparison against the electronic, NMR, EPR, and other spectra of the oligopeptide low molecular weight chromium-binding substance (LMWCr), also examined the ability of the synthetic complexes to activate insulin-stimulated insulin receptor kinase activity. One of the compounds, $[Cr_3O(propionate)_6(H_2O)_3]^+$ or Cr3, was found to activate the receptor in a similar fashion to LMWCr [8]. Cr3 is very water soluble and can be recrystallized from dilute mineral acids so that it should be reasonably stable in the stomach. At a nutritionally relevant level (3 μg Cr/kg body mass) and a pharmacologically relevant level (3 mg/kg), at least 60 and 40% of the compound, respectively, are absorbed in 24 h [14]. This represents a greater than 10-fold increase over those of Cr picolinate (marginally soluble in water, 0.6 mM), $CrCl_3$ (which oligimerizes in water) and Cr nicotinate ('$Cr(nic)_2(OH)$,' insoluble in water). The solubility of Cr3 and its stability, thus, allow a unique amount of the material to enter the circulatory system and tissues.

During the first 24 h after intravenous injection, the fate of the ^{51}Cr-labeled Cr3 in tissues, blood, urine and feces has been followed [15,16]. The complex is readily incorporated into tissues and cells. The complex rapidly disappears from the blood (<30 min) as radiolabeled Cr from Cr3 appears in tissues. In hepatocytes, the intact Cr3 is efficiently transported into microsomes where its concentration reaches a maximum in approximately 2 h (and corresponds to >90% of Cr in the cells from the injected complex); this suggests that Cr3 is actively transported into the cells *via* endocytosis; identification of the protein(s) responsible is needed. As the complex is degraded in hepatocytes and the levels in microsomes rapidly decrease, Cr appears in the urine as LMWCr (or a similar molecular-weight Cr-binding species). The synthetic complex is degraded before or during its disappearance from the microsomes. During the initial periods when the blood concentration of the complex is high, some Cr3 apparently passes into the urine intact [15,16]. These studies identified Cr3 by its elution profile in size exclusion chromatography; thus, a degradation product of Cr3 that contained ^{51}Cr and had a similar molar mass potentially might not be distinguishable from Cr3 in these studies. A technique to specifically identify this ion to follow its fate is needed, and paramagnetic ^2H NMR should serve this purpose.

The effects of Cr3 on healthy rats and rat models of insulin resistance, colorectal cancer and type 1 and type 2 diabetes have been examined where it was found to have beneficial effects on insulin sensitivity, the incidence of colorectal cancer and, in most cases, lipid parameters [17–23]. The compound has been tested for toxic effects *in vivo* [24–28] and *in vitro* [29,30], and activity assays have been performed in cell culture [31–33]. The compound was found to be non-toxic in the *in vivo* assays and to not damage DNA *in vitro*, although the compound could be oxidized by biological oxidants in the absolute absence of reducing agents. When given orally to rats, the LD50 of the cation was estimated to be greater than 2 g/kg body mass [28]. The complex has recently been shown to increase insulin sensitivity in cattle [34], in a very similar effect to previous studies in healthy rodents and rodent models of diabetes. Increases of acute phase response in cattle have also recently been reported [35]. The stability and fate of Cr3 labeled with 2,2-d_2-propionate in water, tissue culture media, blood plasma, and muscle tissue homogenate have been examined by ^2H NMR.

2. Materials and methods

2.1. Materials

$[Cr_3O(O_2CCH_3)_6(H_2O)_3]NO_3$ was prepared as previously described [36]. $[Cr_3O(O_2CCD_3)_6(H_2O)_3]NO_3$ was prepared by the same method using DO_2CCD_3. Cr3 was prepared as previously described [37]. $[Cr_3O(O_2CCD_2CH_3)_6(H_2O)_3]NO_3$ was prepared by the same method using $DO_2CCD_2CH_3$ or $HO_2CCD_2CH_3$. All reagents were used as received. Doubly deionized water was used throughout. Rat muscle tissue was available from previous work. The right leg bundle of vastus lateralis muscle fiber from male Zucker rats was surgically removed, placed in a plastic vial, frozen in liquid nitrogen, and stored at −80 °C. To prepare the homogenate, the tissue was thawed, and a small portion (0.30 g) was homogenized in ice cold homogenization buffer (100 mM Tris, 100 mM $Na_4P_2O_7$, 100 mM NaF, 10 mM EDTA, 10 mM Na_3VO_4, 2 mM PMSF, 1% triton-X, 50 mM β-glycerophosphate, and 10 μg/mL apotinin). Following a 30-minute incubation period, the homogenate was centrifuged at 9100 ×*g* for 10 min at 4 °C. Blood plasma from CD-1 mice was stored at 4 °C prior to use. All work with animals was approved by The University of Alabama Institutional Animal Care and Use Committee. DMEM, Dulbecco's modified Eagle medium, was supplemented to 15 mM HEPES, 20 mM glutamine, 100 units/mL penicillin, 100 units/mL streptomycin, and 10% fetal bovine serum.

2.2. Instrumentation

^2H NMR spectra were obtained on a Bruker AM-500 or Bruker AV-360 spectrometer at approximately 23 °C (23 ± 1 °C). Chemical shifts