Bioinorganic Chemistry

Biomimetic Oxidation of Chromium(III): Does the Antidiabetic Activity of Chromium(III) Involve Carcinogenic Chromium(VI)?**

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The proposed role of Cr^{III} ions as an essential human micronutrient, responsible for insulin activation in glucose and fat metabolism,[1] is currently under dispute.[2,3] The most recent mechanistic hypothesis on the biological activity of the Cr^{III} ion assumes the existence of a specific biomolecule that binds Cr^{III} ions, chromodulin, which enhances interactions of insulin with its cellular receptors.[4] No definitive structural information on chromodulin is available as yet,[4] and its isolation procedure is controversial.[3,5] A well-characterized trinuclear oxo–carboxylato complex, $[Cr^{III}_3O(OCOEt)_6(OH_2)_3]^+$ (**1**)[6] has been proposed as a structural and

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functional mimetic of chromodulin[7] and a safer potential therapeutic agent for Type II diabetes and related disorders[8] than the controversial nutritional supplement, $[Cr^{III}(pic)_3]$ (**2**, pic = picolinato(1−) = pyridine-2-carboxylato(1−)).[9]

Although biological oxidations of Cr^{III} species to genotoxic and carcinogenic Cr^{VI} or Cr^{V} species[3] were previously considered impossible,[10] evidence is mounting for the feasibility of such oxidations under physiologically relevant conditions.[3,11] Herein we propose, for the first time, that the reported insulin-enhancing activities of some Cr^{III} compounds,[1,8] previously attributed to specific interactions of Cr^{III} ions with cellular insulin receptors,[4] are caused by intra- or extracellular oxidations of Cr^{III} to Cr^{V} and/or Cr^{VI} compounds, which act as protein tyrosine phosphatase (PTP) inhibitors by mechanisms similar to those for a well-known insulin mimetic V^{V} species (isoelectronic to Cr^{VI}).[12]

Strong oxidants, such as H_2O_2 and ClO^-, are produced naturally by cells, particularly those involved in the immune response, through the reactions of oxidase enzymes with reductants and O_2.[13] While oxidation of Cr^{III} to Cr^{VI} ions by H_2O_2 in strongly alkaline media is well-known and is widely used in analytical chemistry,[14] only scattered reports exist on the oxidation of some Cr^{III} complexes (with nonbiological ligands) by H_2O_2 or ClO^- in neutral or weakly basic aqueous media (pH 7–9).[15] We chose complex **1** as a main substrate for the systematic studies of the oxidation of Cr^{III} species under physiologically relevant conditions owing to its known biological activity[8] and proposed structural similarity to a purported natural factor that contains Cr^{III} ions.[4,7] Complex **1** can also serve as a representative example of numerous polynuclear species formed on hydrolysis of Cr^{III} complexes in neutral aqueous solutions.[16] It is relatively stable at pH 7.4 and 37 °C, although partial hydrolysis (leading to di- and mononuclear species) occurs on the timescale of hours, as revealed by electrospray mass spectrometry (see the Supporting Information).

Oxidations of **1** by H_2O_2 or ClO^- at pH 7.4 led to the formation of $[Cr^{VI}O_4]^{2-}$, detected from its characteristic absorbance in electronic spectra ($\lambda_{max} = 372$ nm, $\varepsilon_{max} \sim 4.7 \times 10^3\,M^{-1}cm^{-1}$, see the Supporting Information).[14a] Quantitative determination of Cr^{VI} species was performed by the diphenylcarbazide method[17] after the removal of unconverted H_2O_2 by catalase (see the Supporting Information for experimental details), typical results are presented in Table 1.

Micromolar concentrations of Cr^{VI} ions were produced in the reactions of **1** (Cr-ion concentration, 0.10 mM) with sub-mM concentrations of H_2O_2 or ClO^-, as well as with two well-known H_2O_2-producing enzymatic systems, based on glucose oxidase[18] or xanthine oxidase,[19] on the hours timescale at pH 7.0–7.4 and 37 °C (Table 1). This is the first evidence for oxidation of Cr^{III} ions by enzymatic systems. Kinetic studies of the reaction of **1** with the glucose oxidase system showed that the formation of Cr^{VI} species is preceded by the formation of H_2O_2 (see the Supporting Information). Notably, oxidation of **1** by H_2O_2 was efficient in a protein-rich environment and even in undiluted blood serum (buffers *C* and *D* in Table 1). Relatively low but detectable levels of Cr^{VI} species were formed upon the oxidation of **2** or hydrolysed $CrCl_3$ by H_2O_2 (Table 1).[16] No detectable levels of Cr^{V} species (≥ 1 μM, by EPR spectroscopy)[3,11] were formed under the studied conditions (Table 1).

Table 1: Typical conditions and results of Cr^{III} oxidations.

Substrate[a]	Oxidant[b]	Buffer[c]	*t* [h][d]	$[Cr^{VI}]$ [μM][e]
1	H_2O_2 (1.0)	*A*	1.0	12 ± 2
1	H_2O_2 (1.0)	*A*	6.0	33 ± 6
1	H_2O_2 (0.10)	*A*	6.0	4.0 ± 0.7
1	H_2O_2 (1.0)	*B*	6.0	10 ± 1
1	H_2O_2 (1.0)	*C*	6.0	20 ± 1
1	H_2O_2 (1.0)	*D*	1.0[f]	8 ± 2
$CrCl_3$[g]	H_2O_2 (1.0)	*A*	6.0	10 ± 1
2	H_2O_2 (1.0)	*A*	6.0	1.0 ± 0.1
1	ClO^- (0.25)	*B*[h]	6.0	23 ± 2
1	GO[i]	*A*	6.0	14 ± 3
1	XO[j]	*A*	6.0	9 ± 1

[a] In all reactions, $[Cr^{III}]_0 = 0.10$ mM. [b] Concentrations (mM) are given in parentheses. [c] Designations: *A* is 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES, 0.10 M, pH 7.4); *B* is phosphate (0.10 M, pH 7.4); *C* is tris(hydroxymethyl)aminomethane (Tris, 50 mM, pH 7.0), containing NaCl (0.10 M), ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate (EDTA, 0.20 mM), bovine serum albumin (BSA, 1.0 mg mL^{-1}), and Brij 35 (0.001 %); and *D* is fetal bovine serum (undiluted). [d] All the reactions were carried out at 37 °C. [e] Average results and standard deviations of three independent experimental series. [f] Longer reaction times led to partial reduction of the formed Cr^{VI}. [g] Hydrolysis products of $CrCl_3$. [h] No Cr^{VI} formation was observed in buffer *A*, probably because of ClO^- reduction by the buffer. [i] Glucose oxidase (0.10 U mL^{-1}), glucose (5.0 mM), and ambient air O_2. [j] Xanthine oxidase (0.10 U mL^{-1}), xanthine (saturated, ~1 mM), and ambient air O_2.

The abilities of a Cr^{VI} complex, as well as of a model Cr^{V} complex, $[Cr^{V}O(ehba)_2]^-$ (ehba = 2-ethyl-2-hydroxybutanoato(2−)),[20] to inhibit a well-characterized[21] microbial PTP[22] in vitro were comparable to those of V^{V} under the same conditions (Figure 1). The PTP used contained a $Cys(X_5)Arg$ catalytic domain (X is any amino acid), which is common for all the known microbial and mammalian PTPs.[21] The buffer used in the phosphatase reactions (corresponding to *C* in Table 1) contained small amounts of

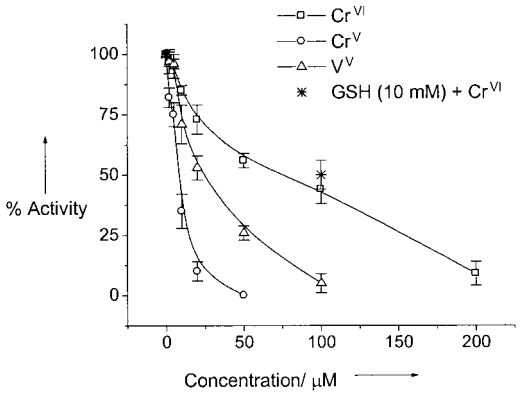

Figure 1. Inhibition of a recombinant *Yersinia enterocolitica* PTP (from Sigma) by Cr^{VI} ions (Na_2CrO_4), Cr^{V} ions ($Na[CrO(ehba)_2]$), or V^{V} ions (Na_3VO_4) in buffer *C* (see note [c] to Table 1), at 37 °C (reaction time 30 min), by using *p*-nitrophenylphosphate (10 mM) as a substrate. Averaged results and standard deviations of three independent experimental series are shown; experimental details are given in the Supporting Information.

thiols ([RSH] $\approx 7\,\mu M$, see the Supporting Information for details), which helped to maintain the active reduced state of the Cys residue in the catalytic domain of the enzyme.[21] To ensure that the inhibitory effects of Cr^{VI} and Cr^{V} specues were not due to the depletion of the antioxidant thiols in the buffer, the abilities of Cr^{VI} and Cr^{V} complexes to reduce the PTP activity in the presence of excess thiol (dithiothreitol, [RSH] = 1.0 mM) were also demonstrated (see the Supporting Information). Furthermore, the efficiency of PTP inhibition by Cr^{VI} species (0.10 mM) was not significantly decreased in the presence of the most abundant biological reductant, glutathione (GSH) at a high physiological concentration (10 mM, Figure 1).[22] These data suggest that Cr^{VI} and Cr^{V} species are likely to react with PTPs even in the thiol-rich intracellular environment. To our knowledge, this is the first example of inhibition of an isolated PTP by Cr^{VI} or Cr^{V} compounds. No significant ($\geq 20\%$) effects on the PTP activity were observed for Cr^{III} compounds (up to 0.50 mM of **1**, **2**, or $CrCl_3$).[23]

Reactions of $[CrO_4]^{2-}$ ions with PTPs probably involve reversible formation of relatively stable five-coordinate thiolato complexes with Cys residues in the catalytic domains of the enzymes (Figure 2),[24] consistent with the generally

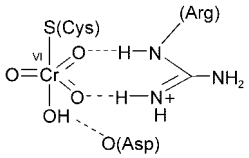

Figure 2. Proposed binding mode of the Cr^{VI} species to the active site of PTP, based on the structures of Cr^{VI} thiolato complexes in aqueous solutions (determined by X-ray absorption spectroscopy)[24] and structures of vanadate-inhibited PTPs (determined by X-ray crystallography).[12b,21b]

accepted mechanism for reactions with $[VO_4]^{3-}$ ions.[12] Reactions of Cr^{V} complexes with Cys residues at the catalytic sites of enzymes, including PTPs, are more likely to lead to their irreversible oxidations,[17b] as was proposed in the mechanism of PTP inhibition by V^{V} peroxo complexes.[12a] Although Cr^{V} species were not detected by EPR spectroscopy during the oxidation of Cr^{III} centers in this work, significant concentrations of such species are likely to form during the reduction of Cr^{VI} centers within the cells or at the cell surface.[3,11] Extracellular oxidation of Cr^{III} to Cr^{VI} ions (e.g., by H_2O_2 or ClO^- released by macrophages)[13] will lead to a dramatic increase in cellular uptake of Cr species.[3,25] Inhibition of PTPs by Cr^{V} and/or Cr^{VI} species is probably responsible for the increased levels of tyrosine phosphorylation in Cr^{VI}-treated cells[26] and for the insulin-mimetic effects of Cr^{VI} species in animals.[27] Such inhibition is likely to lead to disruptions in cell signaling pathways, and to contribute to the Cr^{VI}-induced carcinogenicity.[3]

The relative reactivities of various Cr^{III} complexes towards H_2O_2 (Table 1) may correlate with their reported activities as insulin activators.[1,8] Although both complexes **1** and **2** improve insulin-related metabolic parameters in diabetic rats, only the former complex (which is oxidized more easily, Table 1) is efficient in healthy animals.[8] A stable chelate complex, **2**, is relatively nonreactive in vitro (Table 1); indeed, its stability in gastrointestinal media is one of the main reasons for its use as a nutritional supplement.[1] However, **2** is known to be metabolized by hepatic enzymes with the release of more reactive Cr^{III} species.[28] Rapid hydrolysis of $CrCl_3$ in neutral aqueous media with the formation of insoluble products[16] is a likely reason for its relatively inefficient oxidation to Cr^{VI} (Table 1), as well as for the controversies regarding its biological activity.[1]

In conclusion, the ease of oxidation of **1** to carcinogenic Cr^{VI} under biologically relevant conditions (Table 1) warrants further research into the safety of using **1** (or any other Cr^{III} compound) as a nutritional supplement or therapeutic agent.[8]

Experimental Section

Full details of the used reagents, preparation of reaction solutions and instrumental techniques are given in the Supporting Information.

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