Bioinorganic Chemistry

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

*Irma Mulyani, Aviva Levina, and Peter A. Lay**

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

[*] I. Mulyani, Dr. A. Levina, Prof. P. A. Lay
Centre for Heavy Metals Research
School of Chemistry
University of Sydney
Sydney, NSW 2006 (Australia)
Fax: (+61) 2-9351-3329
E-mail: p.lay@chem.usyd.edu.au

[**] Financial support for this work was provided by an Australian Research Council Discovery grant, including an Australian Professional Fellowship (to P.A.L.). I.M. is grateful to Chemistry Department, Bandung Institute of Technology, Indonesia, for partial funding of her postgraduate studies at the University of Sydney. We thank Dr. Michael Davies (Heart Research Institute, Sydney) for helpful discussions.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/ange.200460113

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

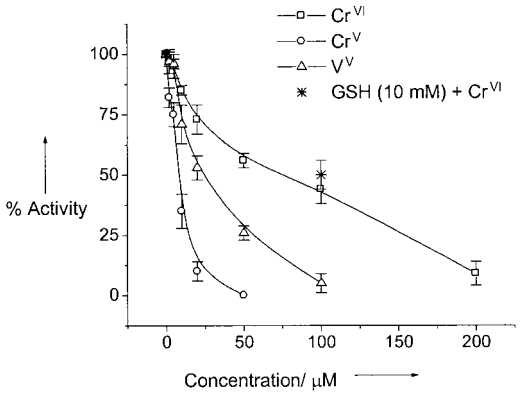

Figure 1. Inhibition of a recombinant *Yersinia enterocolitica* PTP (from Sigma) by Cr^{VI} ions (Na_2CrO_4), Cr^{V} ions ($Na[CrO(ehba)_2]$), or V^{V} ions (Na_3VO_4) in buffer *C* (see note [c] to Table 1), at 37 °C (reaction time 30 min), by using *p*-nitrophenylphosphate (10 mM) as a substrate. Averaged 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

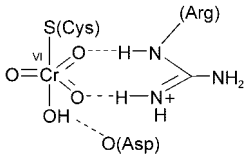

Figure 2. Proposed binding mode of the Cr^{VI} species to the active site of PTP, based on the structures of Cr^{VI} thiolato complexes in aqueous solutions (determined by X-ray absorption spectroscopy)[24] and structures of vanadate-inhibited PTPs (determined by X-ray crystallography).[12b,21b]

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.

Received: March 25, 2004 [Z460113]

Keywords: bioinorganic chemistry · biological activity · chromium · enzyme inhibitors · oxidation

[1] R. A. Anderson, *J. Am. Coll. Nutr.* **1998**, *17*, 548–555.
[2] D. M. Stearns, *BioFactors* **2000**, *11*, 149–162.
[3] A. Levina, R. Codd, C. T. Dillon, P. A. Lay, *Prog. Inorg. Chem.* **2003**, *51*, 145–250, and references therein.
[4] a) C. M. Davis, J. B. Vincent, *Biochemistry* **1997**, *36*, 4382–4385; b) L. Jacquamet, Y. Sun, J. Hatfield, W. Gu, S. P. Cramer, M. W. Crowder, G. A. Lorigan, J. B. Vincent, J.-M. Latour, *J. Am. Chem. Soc.* **2003**, *125*, 774–780; c) J. B. Vincent, *J. Trace Elem. Exp. Med.* **2003**, *16*, 227–236.
[5] E. Gaggelli, F. Berti, N. D'Amelio, N. Gaggelli, G. Valensin, L. Bovalini, A. Paffetti, L. Trabalzini, *Environ. Health Perspect.* **2002**, *110*, 733–738.
[6] A. S. Antsyshkina, M. A. Porai-Koshits, I. V. Arkhangel'skii, I. N. Diallo, *Zh. Neorg. Khim.* **1987**, *32*, 2928–2932; Engl. transl.: A. S. Antsyshkina, M. A. Porai-Koshits, I. V. Arkhangel'skii, I. N. Diallo, *Russ. J. Inorg. Chem.* **1987**, *32*, 1700–1703.
[7] The ability of nM concentrations of **1** to enhance the tyrosine kinase activity of β subunits of insulin receptors has been reported (C. M. Davis, A. C. Royer, J. B. Vincent, *Inorg. Chem.* **1997**, *36*, 5316–5320), but our attempts to reproduce these results were not successful.
[8] a) Y. J. Sun, K. Mallya, J. Ramirez, J. B. Vincent, *J. Biol. Inorg. Chem.* **1999**, *4*, 838–845; b) J. B. Vincent, C. M. Davis (University of Alabama), US Patent 6197816, **2001** [*Chem. Abstr.* **2001**, *134*, 207223]; c) Y. J. Sun, B. J. Clodfelder, A. A. Shute, T. Irvin, J. B. Vincent, *J. Biol. Inorg. Chem.* **2002**, *7*, 852–862.
[9] a) D. M. Stearns, W. H. Armstrong, *Inorg. Chem.* **1992**, *31*, 5178–5184; b) D. M. Stearns, S. M. Silveira, K. K. Wolf, A. M. Luke, *Mutat. Res.* **2002**, *513*, 135–142; c) D. D. D. Hepburn, J. Xiao, S. Bindom, J. B. Vincent, J. O'Donnell, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 3766–3771.
[10] F. L. Petrilli, S. DeFlora, *Mutat. Res.* **1978**, *58*, 167–173.
[11] a) R. Codd, C. T. Dillon, A. Levina, P. A. Lay, *Coord. Chem. Rev.* **2001**, *216–217*, 533–577; b) A. Levina, P. A. Lay, *Coord. Chem. Rev.* **2004**, in press.
[12] a) P. J. Stankiewicz, A. S. Tracey, D. C. Crans in *Metal Ions in Biological Systems*, *Vol. 31* (Eds.: H. Sigel, A. Sigel), Marcel

Dekker, New York, **1995**, pp. 287 – 324; b) M. Zhang, M. Zhou, R. L. Van Etten, C. V. Stauffacher, *Biochemistry* **1997**, *36*, 15 – 23.
[13] B. Halliwell in *Active Oxygen in Biochemistry* (Eds.: J. S. Valentine, C. S. Foote, A. Greenberg, J. F. Liebman), Blackie Academic and Professional, London, **1995**, pp. 313 – 335.
[14] a) G. W. Haupt, *Natl. Bur. Stand. Circ.* **1952**, *48*, 414 – 423; b) J. E. T. Andersen, *Anal. Chim. Acta* **1998**, *361*, 125 – 131.
[15] a) S. C. Pal, M. T. H. Tarafder, *Indian J. Chem. Sect. A* **1996**, *35*, 960 – 963; b) R. Rajan, B. U. Nair, T. Ramasami, *Inorg. React. Mech.* **2000**, *1*, 247 – 255.
[16] P. A. Lay, A. Levina in *Comprehensive Coordination Chemistry II*, *Vol. 4* (Eds.: J. A. McCleverty, T. J. Meyer), Elsevier, Oxford, UK, **2004**, pp. 313 – 413.
[17] a) *Standard Methods for the Examination of Water and Wastewater*, 16th ed., American Public Health Association, Washington, DC, **1985**, Sect. 312B, pp. 201 – 204; b) A. Levina, A. M. Bailey, G. Champion, P. A. Lay, *J. Am. Chem. Soc.* **2000**, *122*, 6208 – 6216.
[18] J. P. Klinman, *J. Biol. Inorg. Chem.* **2001**, *6*,1 – 13, and references therein.
[19] C. Vorbach, R. Harrison, M. R. Capecchi, *Trends Immunol.* **2003**, *24*, 512 – 517, and references therein.
[20] M. Krumpolc, J. Roček, *J. Am. Chem. Soc.* **1979**, *101*, 3206 – 3209. This complex is widely used as a model for the studies of reactions of Cr^{V} complexes reactions biomolecules in vitro.[3,11a]
[21] a) Z.-Y. Zhang, J. C. Clemens, H. L. Schubert, J. A. Stuckey, M. W. F. Fischer, D. M. Hume, M. A. Saper, J. E. Dixon, *J. Biol. Chem.* **1992**, *267*, 23759 – 23766; b) Z.-Y. Zhang, Y. Wang, L. Wu, E. B. Fauman, J. A. Stuckey, H. L. Schubert, M. A. Saper, J. E. Dixon, *Biochemistry* **1994**, *33*, 15266 – 15270.
[22] Reduction of Cr^{VI} to Cr^{III} ions by GSH at physiological conditions is relatively slow (hours timescale) and leads to the formation of reactive Cr^{V}, Cr^{IV}, and organic radical species, which are likely to contribute to PTP inhibition (A. Kortenkamp, M. Casadevall, S. P. Faux, A. Jenner, R. O. J. Shayer, N. Woodbridge, P. O'Brien, *Arch. Biochem. Biophys.* **1996**, *329*, 199 – 207).
[23] The method for determination of phosphatase activity was similar to that described in: B. J. Goldstein, L. Zhu, R. Hager, A. Zilbering, Y. J. Sun, J. B. Vincent, *J. Trace Elem. Exp. Med.* **2001**, *14*, 393 – 404 (see the Supporting Information for details). Opposite effects (activation by $\approx 50\,\%$ or inhibition by $\approx 70\,\%$) of $CrCl_3$ (0.10 mM) on two recombinant human PTPs, described in this reference, are likely to be due to the changes in protein conformation caused by nonspecific binding of Cr^{III} ions.[3] No significant effects of **1** or chromodulin on human PTPs were found, contrary to the earlier reports.[4,7]
[24] A. Levina, P. A. Lay, *Inorg. Chem.* **2004**, *43*, 324 – 335.
[25] C. T. Dillon, P. A. Lay, A. M. Bonin, M. Cholewa, G. J. F. Legge, *Chem. Res. Toxicol.* **2000**, *13*, 742 – 748.
[26] a) E. J. Yurkow, G. Kim, *Mol. Pharmacol.* **1995**, *47*, 686 – 695; b) Y. Qian, B. Jiang, D. C. Flynn, S. S. Leonard, S. W. Wang, Z. Zhang, J. P. Ye, F. Chen, L. Y. Wang, X. L. Shi, *Mol. Cell. Biochem.* **2001**, *222*, 199 – 204.
[27] a) E. Kim, K. J. Na, *Toxicol. Appl. Pharmacol.* **1991**, *110*, 251 – 258; b) Y. Goto, K. Kida, *Jpn. J. Pharmacol.* **1995**, *67*, 365 – 368.
[28] S. A. Kareus, C. Kelley, H. S. Walton, P. R. Sinclair, *J. Hazard. Mater. B* **2001**, *84*, 163 – 174.

