VIP Bioinorganic Chemistry Very Important Paper

International Edition: DOI: 10.1002/anie.201509065
German Edition: DOI: 10.1002/ange.201509065

Carcinogenic Chromium(VI) Compounds Formed by Intracellular Oxidation of Chromium(III) Dietary Supplements by Adipocytes

*Lindsay E. Wu, Aviva Levina, Hugh H. Harris, Zhonghou Cai, Barry Lai, Stefan Vogt, David E. James, and Peter A. Lay**

Abstract: *Chromium(III) nutritional supplements are widely consumed for their purported antidiabetic activities. X-ray fluorescence microscopy (XFM) and X-ray absorption near-edge structure (XANES) studies have now shown that non-toxic doses of $[Cr_3O(OCOEt)_6(OH_2)_3]^+$ (**A**), a prospective antidiabetic drug that undergoes similar H_2O_2 induced oxidation reactions in the blood as other Cr supplements, was also oxidized to carcinogenic Cr^{VI} and Cr^{V} in living cells. Single adipocytes treated with **A** had approximately 1 μm large Cr hotspots containing Cr^{III}, Cr^{V}, and Cr^{VI} (primarily Cr^{VI} thiolates) species. These results strongly support the hypothesis that the antidiabetic activity of Cr^{III} and the carcinogenicity of Cr^{VI} compounds arise from similar mechanisms involving highly reactive Cr^{VI} and Cr^{V} intermediates, and highlight concerns over the safety of Cr^{III} nutritional supplements.*

Chromium(III) supplements are widely consumed for the purported treatment of metabolic disorders, such as insulin resistance and type 2 diabetes.[1] However, controversy exists about both the essentiality of Cr^{III} for humans, and the efficacy and safety of Cr^{III} supplements.[2–5] $[Cr^{III}_3O(OCOEt)_3(OH_2)_3]^+$ (**A** in Figure 1)[6] was proposed as a structural and functional model of the poorly defined Cr^{III} binding peptide, chromodulin, and as an antidiabetic drug.[7] Conversely, we found that chromodulin was an artefact of isolation,[8] and that the biological activity of **A** and other Cr^{III} supplements involved oxidation to genotoxic Cr^{V} and Cr^{VI} in the blood under biologically relevant conditions of oxidative stress.[4,9] We proposed[4] that the insulin-enhancing activities of Cr^{VI} and Cr^{V} have similar mechanisms to those of antidiabetic V^{V}/V^{IV} complexes,[10] namely reversible and/or irreversible binding to cysteines at the active sites of protein tyrosine phosphatases (PTPs) to enhance the insulin signaling cascade.[4,9] The hypothesis that the genotoxicity and carcinogenicity of Cr^{VI}[11] and the controversial antidiabetic activity of Cr^{III}[2—5,12] are based on similar reactive intermediates[4,9] raises safety concerns over Cr^{III} nutritional supplements,[2–4] but evidence for Cr^{VI} in insulin-sensitive cells has not been reported. Herein, we used X-ray fluorescence microscopy (XFM) elemental mapping of single chromium-treated 3T3-L1 adipocytes at submicrometer resolution in combination with microfocus X-ray absorption near-edge structure (μ-XANES) analysis[13–15] of micrometer-sized Cr hotspots to show directly that intracellular oxidation of Cr^{III} does occur.

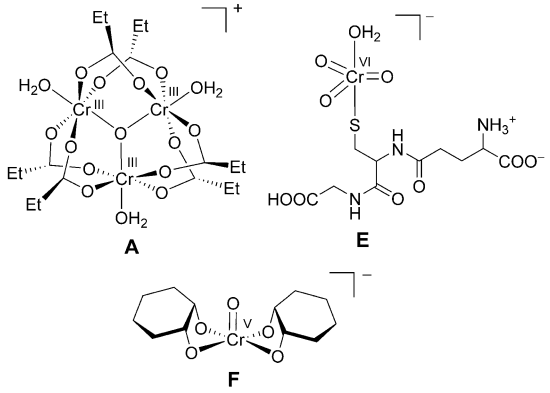

Figure 1. Structures of **A**[6] and the model Cr^{VI} (**E**)[19] and Cr^{V} (**F**)[21] complexes used for XANES data fitting (Figure 3 and Figure 4).

Adipocytes grown on Si_3N_4 substrates[15e] were treated with **A** (100 μM, 20 h, 310 K), then fixed (methanol for ca. 5 s, 253 K), and dried in air. XFM and XANES data were collected at beamline 2-ID-D of the Advanced Photon Source (see the Supporting Information). XFM maps of mature cells showed a relatively low Cr background, punctuated by approximately 1 μm sized hotspots of high Cr intensity (Figure 2; see also the Supporting Information, Figure S1). The maximal density of Cr in the hotspots was 0.17 $\mu g\,cm^{-2}$. By contrast, untreated control samples (Figure S2) showed low background Cr levels (< 0.01 $\mu g\,cm^{-2}$) and no Cr hotspots. Chromium K-edge XANES spectra (spot size: 1×1 μm^2; energy range: 5950–6050 eV; step size: 0.25 eV)[8,9c,15c] were

[*] Dr. L. E. Wu, Dr. A. Levina, Prof. H. H. Harris, Prof. P. A. Lay
School of Chemistry, The University of Sydney
NSW 2006 (Australia)
E-mail: peter.lay@sydney.edu.au
Dr. L. E. Wu, Prof. D. E. James
Garvan Institute of Medical Research
384 Victoria St, Darlinghurst, NSW 2010 (Australia)
Dr. Z. Cai, Dr. B. Lai, Dr. S. Vogt
Advanced Photon Source, X-ray Science Division
Argonne National Laboratory, Argonne, IL 60439 (USA)
Dr. L. E. Wu
Present address: School of Medical Sciences
UNSW Australia, NSW 2052 (Australia)
Prof. H. H. Harris
Present address: School of Chemistry and Physics
The University of Adelaide, SA 5005 (Australia)
Prof. D. E. James
Present address: Charles Perkins Centre
The University of Sydney, NSW 2006 (Australia)
Supporting information and ORCID(s) from the author(s) for this article are available on the WWW under http://dx.doi.org/10.1002/anie.201509065.

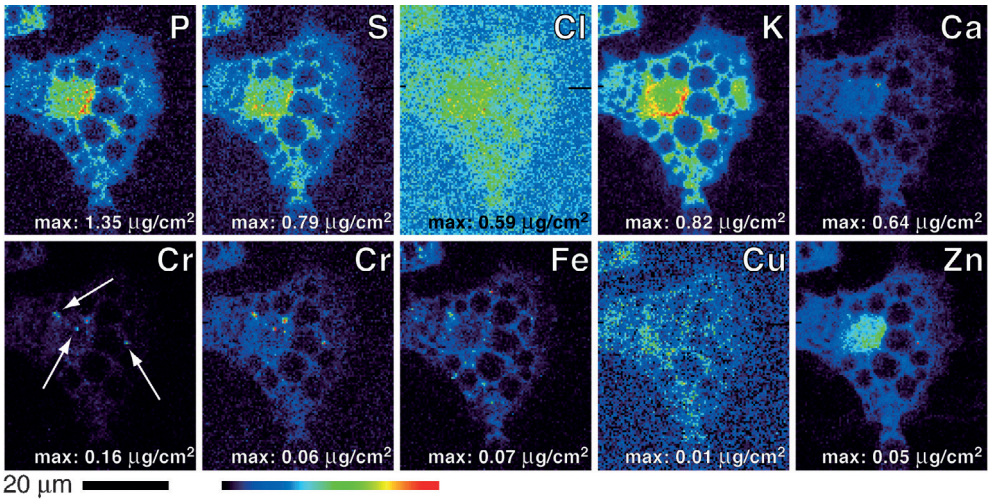

Figure 2. XFM elemental maps (295 K, He atmosphere) of a Cr^{III}-treated (100 μM **A**, 20 h at 310 K) adipocyte with Cr punctate structures (arrows) of unknown identity (maximum concentrations, $\mu g\,cm^{-2}$). A second Cr map is shown with the maximum scaled to 40% to show low-concentration features. The "holes" are X-ray-transparent fat globules that were observed under a microscope.

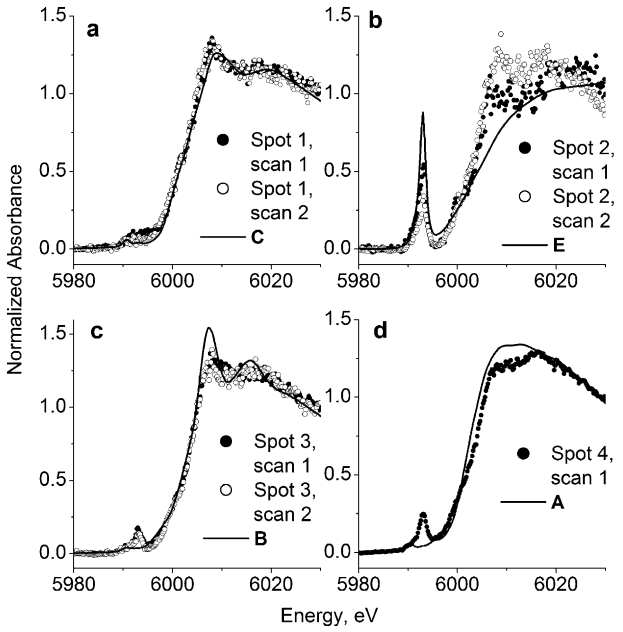

Figure 3. Splined and normalized[17,18] XANES spectra of Cr hotspots ($1\times1\ \mu m^2$, Figure 2, 295 K) in Cr^{III}-treated adipocytes compared with data[8,19] for typical model Cr^{III} and Cr^{VI} complexes (Table 1 and Figure 1). For a color version, see Figure S5.

collected on Cr hotspots from different Cr^{III}-treated cells. The samples were scanned repeatedly to check for photodamage.[16] Figure 3 shows splined and normalized[14,17,18] XANES spectra for single cells, and published[8,19] XANES data for model Cr^{III} and Cr^{VI} complexes (Table 1). All XANES spectra from hotspots had pre-edge bands (symmetry-forbidden 1s→3d transitions)[14a] that were more intense than those of octahedral Cr^{III} complexes (Figure 3). This finding unambiguously confirmed the presence of high oxidation states of Cr ($Cr^{VI}/Cr^{V}/Cr^{IV}$).[14a] The decrease in the pre-edge peak intensities in the second scans (Figure 3a–c) showed X-ray photoreduction of higher oxidation states,[16] and excluded photooxidation of Cr^{III}. Hence, the initial levels of Cr^{VI} and Cr^{V} in the hotspots were higher than those measured.

XANES[8] data from Cr hotspots were fitted to a XANES library of biologically relevant Cr complexes (Table 1).[8,9c,19–24] The best fits (Figure 4; Table S1, Figure S4) included XANES spectra of **A**, its likely hydrolysis products (the Cr^{III} aqua and hydroxido complexes **B** and **C**),[8,9c] and Cr^{III} cysteinato complex **D**.[25] The XANES spectra of other Cr^{III} complexes with amino acid ligands were rejected computationally. The best fits for all single-cell XANES analyses, except for the second scan at spot 1 (Figure 3a), had significant contributions (8–60%, see Table S1) corresponding to the XANES spectrum of a five-coordinate Cr^{VI} glutathione complex (**E**; Table 1, Figure 4),[19] whereas XANES spectra from chromate species[19] were rejected during the fits. The XANES spectrum of a Cr^{V} complex with 1,2-diolato ligands (**F**)[21] contributed only slightly (≤10%, Table S1) to the best fits for spots 3 and 4 (Figure 4 and Table S1). Complex **F** serves as a model of Cr^{V} sugar complexes[26] that have been observed in Cr^{VI}-treated

Table 1: Model Cr complexes used for the XANES fits.

Compound[a]	Ref.[b]	Fit[c]
$[Cr^{III}_3O(OCOEt)_6(OH_2)_3](NO_3)\cdot3H_2O$	[8]	**A**
$Na_9[Cr^{III}(OH)_6]_2(OH)_3\cdot6H_2O$	[8]	**B**
$[Cr^{III}(OH_2)_6](NO_3)_3\cdot3H_2O$	[8]	**C**
$Na[Cr^{III}(cys)_2]\cdot H_2O$	[8]	**D**
$Na[Cr^{VI}O_3(LH_4)(OH_2)]$	[19]	**E**
$Na_2Cr^{VI}O_4\cdot4H_2O$	[19]	–
$Na_3[Cr^{V}O(LH_2)_2]$	[20]	–
$K[Cr^{V}O(chd)_2]$	[21]	**F**
$Na[Cr^{V}O(ehba)]\cdot H_2O$	[22]	–
$[Cr^{IV}O(ehbaH)_2]$	[22]	–
$K[Cr^{V}O(bha)_2]\cdot Me_2CO$	[23]	–
$K_n[Cr(cat)_3]$ $(n=1–3)$[d]	[24]	–

[a] cys = L-cysteinato^{2-}, LH_5 = glutathione;, chd = 1,2-cyclohexanediolato^{2-}, ehba = 2-ethyl-2-hydroxybutanoato^{2-}, bha = benzhydroxamato^{2-}, cat = catecholato^{2-}. [b] References for XANES data. See Table S2 for the references for synthesis and characterization. Published XANES data were re-splined by the method of Penner-Hahn and co-workers[18] as described previously.[9c] [c] Designations of models **A**–**F** used in Figure 4; other model XANES data were rejected computationally.[8] [d] Electrochemically generated reduced and oxidized Cr tris(catecholato) complexes. Oxidation states are ambiguous because of the delocalization of electron density between the Cr center and the ligands.[24]

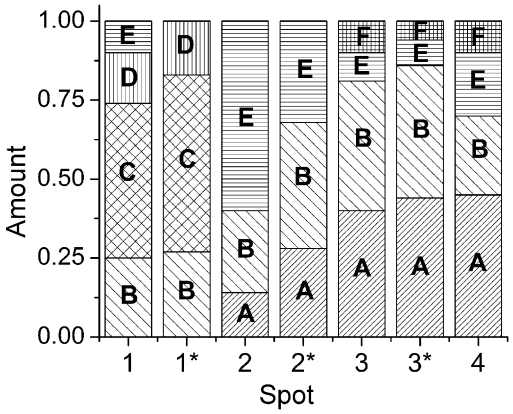

Figure 4. Summary of the best multiple linear regression fits[8,14a] of the XANES spectra from Cr hotspots in Cr^{III}-treated adipocytes (Figure 3; Table S1; Figure S4). The model structures **A**–**F** correspond to those in Table 1 and Figure 1. Asterisks designate a second scan at the same spot (Figure 3). For a color version, see Figure S6.

cells, plants, and animals by EPR spectroscopy.[8,27] Other biologically relevant Cr^{V}[20,22,23] and Cr^{IV} complexes with 2-hydroxycarboxylato[22] or catecholato[24] ligands were rejected computationally during the fitting (Table 1). The oxidation of Cr^{III} to Cr^{VI} in individual adipocytes (Figure 3) is in marked contrast to the reduction of Cr^{VI} to Cr^{III} in other mammalian cells.[8,15a–d] Although intracellular environments are generally reducing, significant local concentrations of strong oxidants, such as H_2O_2, are formed during cell signaling, including insulin signaling,[28] which could be responsible for the observed oxidation of Cr^{III} to Cr^{V} and Cr^{VI} species.[4,9] The formation of Cr^{VI} thiolato species (modelled by compound **E**)[19] as the most abundant product of Cr^{III} oxidation in adipocytes (Figure 4) is consistent with the binding of Cr^{VI} to cysteine residues in the active centers of PTPs.[4,9] XANES fits (Figure 4) indicated the presence of significant amounts of **A**, despite its low stability in cell culture medium.[9c] These results may point to the rapid uptake of **A** by endocytosis,[29] which could explain the observed punctate Cr distributions in cells (Figures 2 and S1), but the nature of these structures is unclear. As observed previously,[30] there was punctate distribution of Fe (Figures 2, S1, and S2), possibly owing to transferrin-mediated uptake of Fe^{III} into endosomes. Deposits of xenobiotic elements in punctate areas have previously been observed in XFM studies of Cr^{VI}-treated cells,[15d] and with supraphysiological concentrations of Se^{IV}, Ti^{IV}, and V^{IV} species.[31,32] In spot 1 (Figures 3 and 4), **A** was replaced with Cr^{III} hydrolysis products (**B** and **C**)[8] and with a Cr^{III} cysteinato complex (**D**), which is a model of Cr^{VI} thiolato reduction products.[33] As spot 1 was more photoreduced than the other three spots (Figure 3), it is likely that the hydrolysis of **A** with formation of **B** and **C** was catalyzed by partial photoreduction of kinetically inert Cr^{III} to reactive Cr^{II} species.[34] Lower concentrations of hydrolysis product **B** were also present in other hotspots (Figure 4).

In summary, XFM and XANES data from chromium(III)-treated adipocytes provide strong support for the hypothesis that the antidiabetic activity of Cr^{III} complexes is based on the formation of reactive, and carcinogenic, Cr^{V} and Cr^{VI} intermediates.[4,9] This raises concern over the possible carcinogenicity of Cr^{III} compounds[2,35] and the risks of long-term Cr^{III} nutritional supplementation.[4] Although animal experiments have yet to provide conclusive evidence for Cr^{III} carcinogenicity,[11] these studies cannot be extrapolated to human exposure because of the long latency time of chromium-induced cancer in humans,[11] and the long-term exposure of patients with diabetes to the oxidative stress that facilitates Cr oxidation in both the blood[9] and cells. Animal studies that mimic long-term oxidative stress have yet to be conducted. In light of these findings, there is a need for epidemiological studies to ascertain whether Cr^{III} supplements alter cancer risk.

Acknowledgements

Financial support for this work was provided by the Australian Research Council (to P.A.L.) and the Australian Synchrotron Research Program (ASRP). We thank the ASRP for an ASRP Research Fellowship (H.H.H.) and for access to APS and ANBF facilities (Photon Factory, Tsukuba, Japan). The ASRP is funded by the Commonwealth of Australia under the Major National Research Facilities Program. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory (DE-AC02-06CH11357). L.E.W. is a Cancer Institute NSW Early Career Fellow.

Keywords: adipocytes · cancer · chromium · oxidation · X-ray fluorescence microscopy

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 1742–1745
Angew. Chem. **2016**, *128*, 1774–1777

[1] a) *The Nutritional Biochemistry of Chromium(III)* (Ed.: J. B. Vincent), Elsevier, Amsterdam, **2007**; b) P. Mason, *Dietary Supplements*, 4th ed., Pharmaceutical Press, London, **2011**.
[2] a) D. M. Stearns, *BioFactors* **2000**, *11*, 149–162; b) D. M. Stearns in *The Nutritional Biochemistry of Chromium(III)* (Ed.: J. B. Vincent), Elsevier, Amsterdam, **2007**, pp. 57–70 and 209–224.
[3] a) J. B. Vincent, *Dalton Trans.* **2010**, *39*, 3787–3794; b) J. B. Vincent, *The Bioinorganic Chemistry of Chromium*, Wiley, Chichester, UK, **2013**; c) J. B. Vincent, *J. Trace Elem. Med. Biol.* **2014**, *28*, 397–405.
[4] a) A. Levina, P. A. Lay, *Chem. Res. Toxicol.* **2008**, *21*, 563–571; b) A. Levina, P. A. Lay, *Dalton Trans.* **2011**, *40*, 11675–11686; c) P. A. Lay, A. Levina in *Binding, Transport and Storage of Metal Ions in Biological Cells* (Eds.: W. Maret, A. Wedd), Royal Society of Chemistry, London, **2014**, pp. 188–222.
[5] C. H. Bailey, *Biol. Trace Elem. Res.* **2014**, *157*, 1–8.
[6] A. S. Antsyshkina, M. A. Porai-Koshits, I. V. Arkhangel'skii, I. N. Diallo, *Russ. J. Inorg. Chem.* **1987**, *32*, 1700–1703.
[7] a) C. M. Davis, A. C. Royer, J. B. Vincent, *Inorg. Chem.* **1997**, *36*, 5316–5320; b) J. B. Vincent, C. M. Davis, US Patent US 6197816, **2001**; c) Y. J. Sun, B. J. Clodfelder, A. A. Shute, T. Irvin, J. B. Vincent, *J. Biol. Inorg. Chem.* **2002**, *7*, 852–862.
[8] A. Levina, H. H. Harris, P. A. Lay, *J. Am. Chem. Soc.* **2007**, *129*, 1065–1075.
[9] a) I. Mulyani, A. Levina, P. A. Lay, *Angew. Chem. Int. Ed.* **2004**, *43*, 4504–4507; *Angew. Chem.* **2004**, *116*, 4604–4607; b) A.

Levina, I. Mulyani, P. A. Lay in *The Nutritional Biochemistry of Chromium(III)* (Ed.: J. B. Vincent), Elsevier, Amsterdam, **2007**, pp. 225 – 256; c) A. Nguyen, I. Mulyani, A. Levina, P. A. Lay, *Inorg. Chem.* **2008**, *47*, 4299 – 4309.
[10] a) K. Dralle Mjos, C. Orvig, *Chem. Rev.* **2014**, *114*, 4540 – 4563; b) Y. Yoshikawa, H. Sakurai, D. C. Crans, G. Micera, E. Garriba, *Dalton Trans.* **2014**, *43*, 6965 – 6972; c) D. Rehder, *Future Med. Chem.* **2012**, *4*, 1823 – 1837.
[11] International Agency for Research on Cancer. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 100. A Review of Human Carcinogens. Part C: Arsenic, Metals, Fibres, and Dusts. IARC, Lyon, France, **2012**.
[12] a) G. Chen, P. Liu, G. R. Pattar, L. Tackett, P. Bhonagiri, A. B. Strawbridge, J. S. Elmendorf, *Mol. Endocrinol.* **2006**, *20*, 857 – 870; b) P. Mackowiak, Z. Krejpcio, M. Sassek, P. Kaczmarek, I. Hertig, J. Chmielewska, T. Wojciechowicz, D. Szczepankiewicz, D. Wieczorek, H. Szymusiak, K. W. Nowak, *Mol. Med. Rep.* **2010**, *3*, 347 – 353.
[13] T. Paunescu, S. Vogt, J. Maser, B. Lai, G. Woloschak, *J. Cell. Biochem.* **2006**, *99*, 1489 – 1502.
[14] a) J. B. Aitken, A. Levina, P. A. Lay, *Curr. Top. Med. Chem.* **2011**, *11*, 553 – 571; b) A. A. Hummer, A. Rompel, *Metallomics* **2013**, *5*, 597 – 614.
[15] a) C. T. Dillon, P. A. Lay, M. Cholewa, G. J. F. Legge, A. M. Bonin, T. J. Collins, K. L. Kostka, G. Shea-McCarthy, *Chem. Res. Toxicol.* **1997**, *10*, 533 – 535; b) N. Kitamura, A. M. Ektessabi, *J. Synchrotron Radiat.* **2001**, *8*, 981 – 983; c) R. Ortega, B. Fayard, M. Salomé, G. Devès, J. Susini, *Chem. Res. Toxicol.* **2005**, *18*, 1512 – 1519; d) H. H. Harris, A. Levina, C. T. Dillon, I. Mulyani, B. Lai, Z. Cai, P. A. Lay, *J. Biol. Inorg. Chem.* **2005**, *10*, 105 – 118; e) E. A. Carter, B. S. Rayner, A. I. McLeod, L. E. Wu, C. P. Marshall, A. Levina, J. B. Aitken, P. K. Witting, B. Lai, Z. Cai, S. Vogt, Y.-C. Lee, C.-I. Chen, M. J. Tobin, H. H. Harris, P. A. Lay, *Mol. Biosyst.* **2010**, *6*, 1316 – 1322.
[16] G. N. George, I. J. Pickering, M. J. Pushie, K. Nienaber, M. J. Hackett, I. Ascone, B. Hedman, K. O. Hodgson, J. B. Aitken, A. Levina, C. Glover, P. A. Lay, *J. Synchrotron Radiat.* **2012**, *19*, 875 – 886.
[17] a) P. J. Ellis, H. C. Freeman, *J. Synchrotron Radiat.* **1995**, *2*, 190 – 195; b) *XFit for Windows*, *beta-version*. Australian Synchrotron Research Program, Sydney, Australia, **2004**.
[18] T.-C. Weng, G. S. Waldo, J. E. Penner-Hahn, *J. Synchrotron Radiat.* **2005**, *12*, 506 – 510.
[19] A. Levina, P. A. Lay, *Inorg. Chem.* **2004**, *43*, 324 – 335.
[20] A. Levina, L. Zhang, P. A. Lay, *Inorg. Chem.* **2003**, *42*, 767 – 784.
[21] R. Bartholomäus, K. Harms, A. Levina, P. A. Lay, *Inorg. Chem.* **2012**, *51*, 11238 – 11240.
[22] A. Levina, R. Codd, G. J. Foran, T. W. Hambley, T. Maschmeyer, A. F. Masters, P. A. Lay, *Inorg. Chem.* **2004**, *43*, 1046 – 1055.
[23] S. Gez, R. Luxenhofer, A. Levina, R. Codd, P. A. Lay, *Inorg. Chem.* **2005**, *44*, 2934 – 2943.
[24] A. Levina, G. J. Foran, D. I. Pattison, P. A. Lay, *Angew. Chem. Int. Ed.* **2004**, *43*, 462 – 465; *Angew. Chem.* **2004**, *116*, 468 – 471.
[25] P. De Meester, D. J. Hodgson, H. C. Freeman, C. J. Moore, *Inorg. Chem.* **1977**, *16*, 1494 – 1498.
[26] a) R. Codd, J. A. Irwin, P. A. Lay, *Curr. Opin. Chem. Biol.* **2003**, *7*, 213 – 219; b) R. Bartholomäus, J. A. Irwin, L. Shi, S. Meejoo Smith, A. Levina, P. A. Lay, *Inorg. Chem.* **2013**, *52*, 4282 – 4292.
[27] a) K. J. Liu, X. Shi, J. Jiang, F. Goda, N. Dalal, H. M. Swartz, *Ann. Clin. Lab. Sci.* **1996**, *26*, 176 – 184; b) K. J. Appenroth, M. Bischoff, H. Gabrys, J. Stoeckel, H. M. Swartz, T. Walczak, K. Winnefeld, *J. Inorg. Biochem.* **2000**, *78*, 235 – 242.
[28] a) B. J. Goldstein, K. Mahadev, X. Wu, L. Zhu, H. Motoshima, *Antioxid. Redox Signaling* **2005**, *7*, 1021 – 1031; b) T. Finkel, *J. Cell Biol.* **2011**, *194*, 7 – 15.
[29] N. R. Rhodes, P. A. LeBlanc, J. F. Rasco, J. B. Vincent, *Biol. Trace Elem. Res.* **2012**, *148*, 409 – 414.
[30] P. K. Witting, H. H. Harris, B. S. Rayner, J. B. Aitken, C. T. Dillon, R. Stocker, B. Lai, Z. Cai, P. A. Lay, *Biochemistry* **2006**, *45*, 12500 – 12509.
[31] C. M. Weekley, J. B. Aitken, S. Vogt, L. A. Finney, D. J. Paterson, M. D. de Jonge, D. L. Howard, P. K. Witting, I. F. Musgrave, H. H. Harris, *J. Am. Chem. Soc.* **2011**, *133*, 18272 – 18279.
[32] J. B. Waern, H. H. Harris, B. Lai, Z. Cai, M. M. Harding, C. T. Dillon, *J. Biol. Inorg. Chem.* **2005**, *10*, 443 – 452.
[33] P. A. Lay, A. Levina, *Inorg. Chem.* **1996**, *35*, 7709 – 7717.
[34] A. D. Kirk, *Chem. Rev.* **1999**, *99*, 1607 – 1640.
[35] Z. Fang, M. Zhao, H. Zhen, L. Chen, P. Shi, Z. Huang, *PlosOne* **2014**, *9*, e103194.

Received: September 27, 2015
Published online: December 22, 2015

