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Biomimetic Oxidation of Chromium(III). Does the Anti-Diabetic Activity of Chromium(III) Involve Carcinogenic Chromium(VI)?

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Experimental Section

Caution. Cr^{VI} compounds are human carcinogens, and Cr^{V} complexes are mutagenic and potentially carcinogenic.[3] Contact with skin and inhalation must be avoided.

Reagents. The following enzymes from Sigma were used as received: a recombinant protein tyrosine phosphatase (Yop51*) from *Yersinia enterocolitica* that contains the C235R mutation for enhanced stability; glucose oxidase (EC 1.1.3.4) from *Aspergillius niger*; xanthine oxidase (EC 1.1.3.22) from bovine milk; and catalase (EC 1.11.1.6) from bovine liver. The following commercial reagents of analytical or higher purity grade were used without further purification: *N*,*N*-dimethylformamide (DMF, HPLC grade), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), 2-ethyl-2-hydroxybutanoic acid ($ehbaH_2$), picolinic (pyridine-2-carboxylic) acid ($picH_2$), propionic anhydride, MeOH (HPLC grade), NaOCl aqueous solution, NH_4OAc, $Na_2CrO_4{\cdot}4H_2O$, and NaOH (99.9%) from Aldrich; L-ascorbic acid from BDH biochemicals; tris(hydroxymethyl)aminomethane (Tris) and Chelex 100 resin from BioRad; *sym*-diphenylcarbazide from Fluka; fetal bovine serum (tissue culture grade) from Gibco; ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid disodium salt (Na_2EDTA), D-glucose, $CrCl_3{\cdot}6H_2O$, $Cr(NO_3)_3{\cdot}9H_2O$, $FeSO_4{\cdot}7H_2O$, H_2O_2 (30% aqueous solution), HCl (37% aqueous solution, trace pure), HNO_3 (69% aqueous solution, trace pure), H_2SO_4, $NaH_2PO_4{\cdot}H_2O$, NH_3 (32% aqueous solution), and NaCl from Merck; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) from Research Organics; and bovine serum albumin (BSA), Brij 35, *p*-nitrophenylphosphate, sodium orthovanadate (≥ 90% Na_3VO_4), xanthine and xylenol orange from Sigma. Water was purified by the Milli-Q technique. The Cr^{III} complexes, $[Cr_3O(OCOEt)_6(OH_2)_3](NO_3){\cdot}2H_2O$ (**1**) and $[Cr(pic)_3]{\cdot}H_2O$ (**2**), were synthesized by literature methods,[6,9a] and their purity was confirmed by elemental analyses and electrospray mass spectrometry (for **1**, Figure S1).[S1] The following results of elemental analyses were obtained (calculated values are given in

parentheses): **1** ($Cr_3C_{18}H_{40}NO_{21}$) Cr, 20.1 (20.5); C, 28.9 (28.3), H, 4.98 (5.21), and N, 1.77 (1.84); and **2** ($CrC_{18}H_{14}N_3O_7$) Cr, 11.5 (11.9), C, 48.9 (49.5), H, 2.94 (3.23), and N, 9.70 (9.63). The Cr^V complex, $Na[CrO(ehba)_2]\cdot 1.5H_2O$, was synthesized by a literature method,[20,S2] and its purity was confirmed by electronic and EPR spectroscopies (for 1.0 mM solution in DMF, $\varepsilon_{510} = 168$ M^{-1} cm^{-1}, $g_{iso} = 1.9783$, and $A_{iso}(^{53}Cr) = 17.1\times10^{-4}$ cm^{-1}).[20,S2]

General Methods. Stock solutions used for preparation of the buffers (1.0 M of HEPES, Tris, NaH_2PO_4, or NH_4OAc) were treated with Chelex 100 chelating resin and stored at 4 °C. Working solutions of the buffers were prepared daily by adjusting the pH values of the stock solutions with high purity NaOH, HCl or NH_3; the pH values were measured by an Activon 210 ionometer with an AEP 321 glass/calomel electrode. Concentrations of the catalytic metals (Fe^{III} and Cu^{II}) in the working buffers, determined by Buettner's ascorbate method,[S3] were < 0.5 μM. Working solutions of the enzymes were prepared daily and kept on ice.

All the reactions were performed at 37 ± 0.2 °C, using a Grant LTD 6G thermostat, and the results were reproduced in at least two independent experimental series, using different sets of stock solutions. A Hewlett Packard HP8452A diode-array spectrophotometer was used for electronic spectroscopic measurements. Calibrations for spectrophotometric assays were performed by a standard addition method. Electrospray mass spectrometry (ESMS) was performed using a Finnigan LCQ mass spectrometer; typical experimental settings were as follows: sheath gas (N_2) pressure, 60 psi; spray voltage, 4.0 kV; capillary temperature, 200 °C; capillary voltage, 15 V; tube lens offset, 30 V; *m*/*z* range, 50-2000; scan time, 10 ms (average of ≥10 scans); and carrier solution, MeOH/H_2O (1:1 v/v, flow rate 0.20 mL min^{-1}). X-band EPR spectra were recorded using a flat quartz cell on a Bruker EMX spectrometer equipped with a Bruker EMX 035M NMR gaussmeter and a Bruker EMX 048T microwave frequency counter. The instrument settings were as follows: center field, 3480 G; sweep width, 200 G; resolution, 1024 points; microwave frequency, ~9.67 GHz; microwave power, 20 mW; modulation frequency 100 kHz; modulation amplitude, 1.0 G, and scan time, 21 s (average of 10 scans). Spectra were processed using WIN-EPR software,[S4] second-order corrections were applied to the estimations of EPR parameters. Determinations of Cr in **1** and **2** were performed by atomic absorption spectroscopy (a Varian AA800 spectrometer with C_2H_2/air flame atomization) after digestion of the samples with 69% HNO_3. Determinations of C, H, and N in **1** and **2** were performed by Microanalytical Services, Research School of Chemistry, Australian National University (Canberra), using a Carlo Erba 1106 automatic analyzer.

Oxidations of Cr^{III} Complexes. Stock solutions of **1** (50 mM Cr in H_2O), **2** (10 mM in DMF), and $CrCl_3$ (50 mM in H_2O) were prepared daily. Stock solutions of H_2O_2 and NaClO (~0.5 M) were stored at 4 °C and standardized by iodometric titration[S5] and electronic spectroscopy ($\varepsilon_{292} = 350$ M^{-1} cm^{-1} at pH 12),[S6] respectively. The absence of significant influence of DMF on the oxidation of **2** was checked by the additions of DMF (up to 5% vol.) to the reaction solutions. Reactions of Cr^{III} with H_2O_2 or NaOCl were carried out in closed Eppendorf tubes (maximal volume 1.5 mL), and the reactions with enzymatic systems were carried out in open plastic tubes (maximal volume 5.0 mL), to assist the O_2 diffusion into the reaction solutions; in all cases, the reaction volumes were 1.0 mL. The reactions with H_2O_2 or enzymatic systems were stopped by the addition of catalase (50 μg mL^{-1}).[S7] Preliminary experiments showed that no Cr^{VI} was formed under the conditions of Table 1 (main text), when ≥0.5 μg mL^{-1} catalase was added at the beginning of the

reaction. Quenching was not required for the reaction of **1** with NaOCl, since no active Cl was left in solution after 6 h of reaction at 37 °C (Table 1, checked by iodometric titration).[S5] Formation of Cr^{VI} was first assessed qualitatively from the absorbance of $[CrO_4]^{2-}$ at 372 nm ($\varepsilon \sim 4.7\times10^3$ M^{-1} cm^{-1} at pH 7.4);[14a,S8] the absorbances of Cr^{III} complexes at this wavelength were negligibly low (Figure S2). Quantitative determination of Cr^{VI} by this method was complicated in some cases (particularly for the oxidation of $CrCl_3$) by the formation of insoluble hydrolysis products. Determination of Cr^{VI} for all the reactions (Table 1) was performed by a modified diphenylcarbazide method.[17] Typically, a solution of HCl (0.50 M, 0.50 mL) was added to the reaction solution (0.50 mL), followed by the addition of diphenylcarbazide solution (10 μL, 40 mM in DMF), and the absorbance at 540 nm was measured after 2 min (22 ± 1 °C, $\varepsilon = 4.2\times10^4$ M^{-1} cm^{-1}). Consistent results were obtained from the determinations of Cr^{VI} by the both methods. Formation of H_2O_2 during the reaction of **1** with the glucose oxidase system (Figure S3) was followed by a xylenol orange assay.[S9] In a typical assay, the reaction mixture, or the buffer solution for a blank measurement (10 μL), were added to an aqueous solution (0.99 mL), containing xylenol orange (0.20 mM), Fe^{II} (0.50 mM), and H_2SO_4 (25 mM), and the absorbance at 560 nm was measured after 30 min (22 ± 1 °C, $\varepsilon = 4.8\times10^4$ M^{-1} cm^{-1}). The presence of Cr(VI) (>10 μM) in the reaction mixtures led to significant increases in the absorbance values.

Phosphatase Inhibition Assays. The activity of Yop51* protein tyrosine phosphatase (PTP) was measured by its ability to hydrolyse *p*-nitrophenylphosphate (PNPP, 10 mM) to *p*-nitrophenol ($\varepsilon_{405} = 1.8\times10^4$ M^{-1} cm^{-1} at pH ≥ 12).[23] The reaction components were added on ice to the reaction buffer (buffer *C* in Table 1, 43 μL) in the following order: dilute enzyme solution (1.0 μL in buffer *C*), inhibitor (1.0 μL of aqueous solution), and PNPP (5.0 μL of 0.10 M aqueous solution). Stock solutions of $[Cr^{V}O(ehba)_2]^-$ in H_2O were prepared at <15 min before use and kept on ice.[17b] Stock solutions of $[VO_4]^{3-}$ (10 mM) contained added NaOH (10 mM).[S10] After incubation at 37 °C for 30 min, the reactions were stopped by the addition of NaOH (50 μL, 0.10 M) to the reaction mixtures on ice, and the absorbances at 404 nm were measured immediately. For each experiment, a separate blank solution was used, which contained all the components of the reaction mixture (including Cr and V compounds), except for the enzyme, and was treated in the same way as the reaction mixture. Inclusion of Cr^{VI} or Cr^{V} compounds into the blanks was necessary since these compounds possess significant absorbances at ~400 nm.[17b,S7] Typically, 0.20 U enzyme per reaction was used, and this led to $A_{404} = 1.0 \pm 0.3$ ($l = 1$ cm) in the absence of inhibitors. Since the absolute values of A_{404} varied significantly between different preparations of the dilute enzyme, the experiments were performed in series, each of them included the reactions in the absence or presence of an inhibitor. Relative PTP activities varied by ≤10% between the parallel experimental series (Figure 1 in the main text).

The buffer used for the PTP reactions, as well as for dilutions of the enzyme, corresponded to that recommended by Sigma (buffer *C* in Table 1, 50 mM Tris-HCl, pH 7.0, containing 0.10 M NaCl, 0.20 mM Na_2EDTA, 1.0 mg mL^{-1} BSA, and 0.001% Brij 35),[S11] but it did not contain (except for the experiments in Figure S4) added dithiothreitol (DTT, 2*R*,3*R*-1,4-dimercapto-2,3-butanediol), a strong reductant usually used to maintain the active reduced state of the Cys residue in the catalytic domain of the enzyme.[23] However, DTT had to be added to the concentrated solution of the enzyme (57 U μl^{-1}, supplied by the manufacturer), to restore its activity, which was

lost on storage (2-3 months at –70 °C). The PTP activity was fully restored after the reaction with DTT (5.0 mM) in buffer *C* for 3 h at 0 °C. Thus, the reaction buffer contained thiolato groups, originating from DTT added to the concentrated enzyme, from the enzyme itself, and from BSA. Total concentrations of thiols in the reaction mixtures ([RSH] ~ 7 μM) were determined with Ellman's reagent,[S12] as described previously.[17b] For most of the PTP reactions, no attempts were made to exclude O_2 from the reaction medium. Some of the reactions were performed in Ar-saturated solutions with all the manipulations carried out under a stream of oxygen-free N_2, but these changes did not significantly affect the PTP activity. Addition of DTT (0.50 mM) to the reaction mixture (in the absence of inhibitors) increased the PTP activity by only ~20% (Figure S4).

Incubations of Cr^{VI} (Na_2CrO_4, 10-200 μM) with the reaction buffer in the presence of PNPP, but in the absence of enzyme, for 30 min at 37 °C, did not lead to a significant reduction of Cr^{VI} (determined from the intensity of its absorbance at 372 nm).[14b,S8] Reactions of $[Cr^{V}O(ehba)_2]^-$ under the same conditions led to complete disproportionation of Cr^{V} with the formation of Cr^{VI} and Cr^{III} in a 2:1 molar ratio.[S13] Similar reactions in the presence of added DTT (0.50 mM, [RSH] = 1.0 mM) led to a partial or full reduction to Cr^{III} for Cr^{VI} and Cr^{V}, respectively (see the caption to Figure S4).

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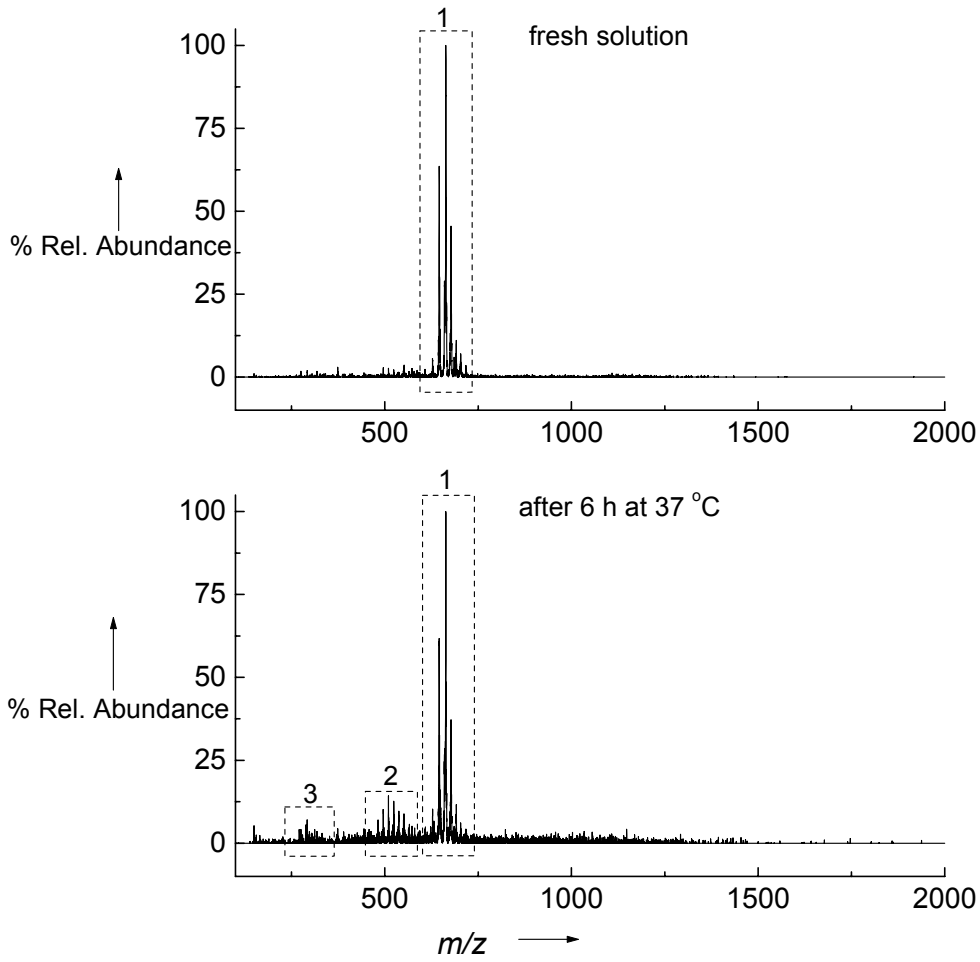

Figure S1. Typical results of elecrospray mass spectrometry (ESMS, positive-ion mode) for **1** ([Cr] = 0.10 mM) in aqueous NH_4OAc/NH_3 buffer (10 mM, pH 7.4), containing MeOH (~10% vol.). Signals 1 are due to the $[Cr_3O(OCOEt)_6(OHR)_x]^+$ species (R = H or Me, x = 0-3), formed from the parent $[Cr_3O(OCOEt)_6(OH_2)_3]^+$ ion under ESMS conditions,[S1] and signals 2 and 3 are due to di- and mononuclear Cr^{III} complexes, respectively. No significant signals were detected in the negative-ion mode (–*m/z* = 50-2000). Such experiments could not be performed in buffers *A*-*D* (Table 1 in the main text), due to their incompatibility with ESMS.

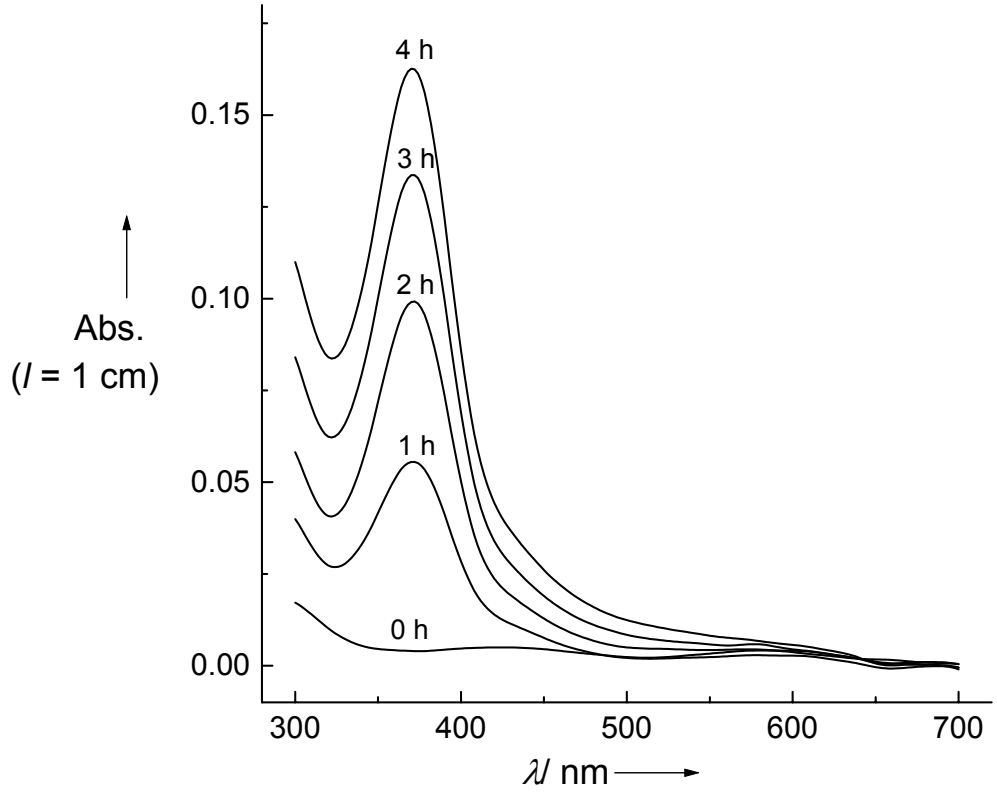

Figure S2. Changes in electronic spectra of the reaction mixtures during the oxidation of **1** ([Cr] = 0.10 mM) with H_2O_2 (1.0 mM) in HEPES buffer (0.10 M, pH 7.4) at 37 ^{o}C.

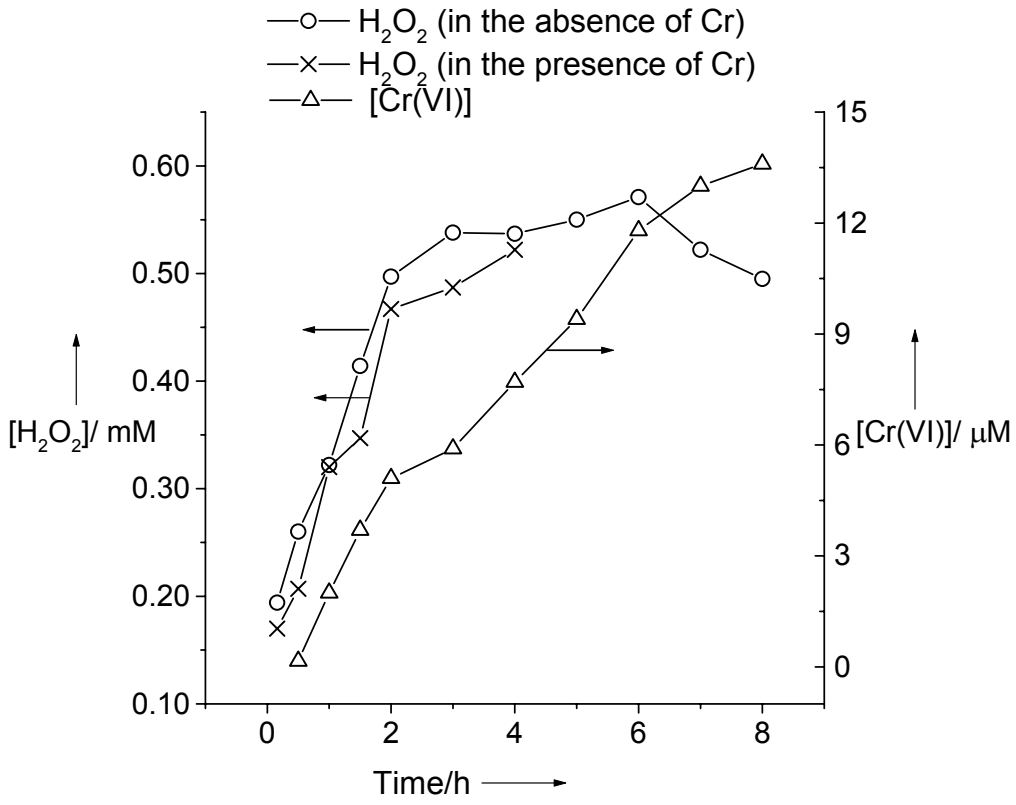

Figure S3. Formation of H_2O_2 (determined by xylenol orange assay)[S9] and Cr(VI) (determined by diphenylcarbazide assay after the removal of H_2O_2 by 50 μg mL^{-1} catalase)[17] during the reaction of **1** ([Cr] = 0.10 mM) with glucose oxidase (0.10 U ml^{-1}), glucose (5.0 mM) and O_2 (ambient air) in HEPES buffer (0.10 M, pH 7.4) at 37 ^{o}C. Formation of H_2O_2 under the same conditions in the absence of **1** is shown for comparison. The results of H_2O_2 determination at $t > 4$ h were significantly affected by the formed Cr^{VI}, these data are not shown.

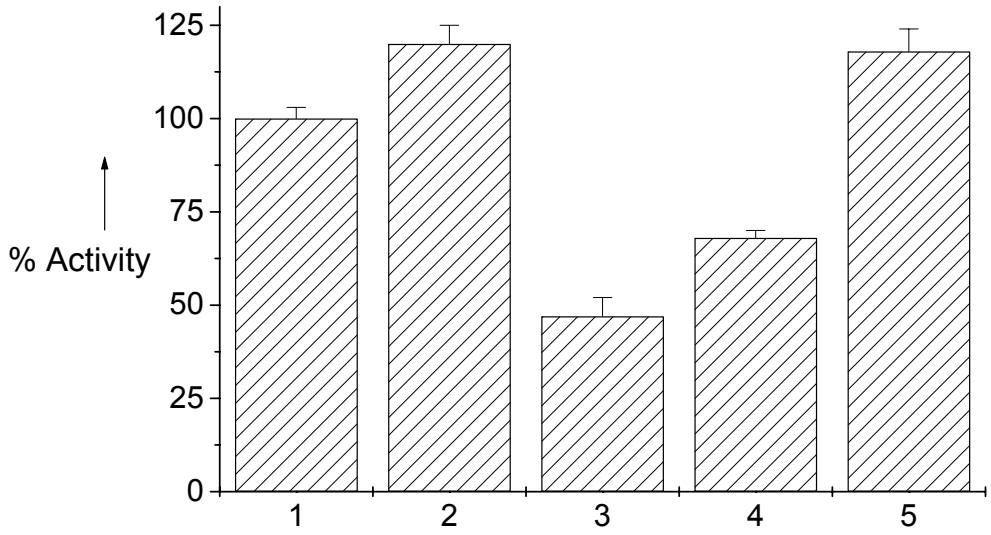

Figure S4. Inhibition of a recombinant *Yersinia enterocolitica* PTP by Cr^{VI} (Na_2CrO_4, 0.10 mM) or Cr^{V} ($Na[CrO(ehba)_2]$, 0.10 mM) in buffer *C* (corresponds to Table 1 and Figure 1 in the main text) in the presence of DTT (0.50 mM, $[RSH]_0$ = 1.0 mM) at 37 °C (reaction time 30 min), using *p*-nitrophenylphosphate (10 mM) as a substrate. Variations in the reaction conditions: (1) no DTT, no Cr; (2) DTT, no Cr; (3) DTT + Cr^{VI}; (4) DTT + Cr^{V}; (5) Cr^{VI} was fully reduced to Cr^{III} by DTT (2 h at 37 °C, determined by disappearance of the Cr^{VI} signal at λ_{max} = 372 nm)[17,S13] before the phosphatase reaction was started. Averaged results and standard deviations of three independent experimental series are shown. Reactions of Cr^{VI} or Cr^{V} with DTT under the conditions (3) or (4), respectively, led to the reduction of ~75% of Cr^{VI} or 100% of Cr^{V} to Cr^{III} (reaction time 30 min; determined from the intensity of the Cr^{VI} signals at 372 nm).[17,S13] Concentrations of DTT at the end of the phosphatase reactions under the conditions (2)-(5) were at 60-95% of the initial value (determined with Ellman's reagent);[17b,S12] thus, excess DTT was present throughout the reactions.