# A residue outside the active site CXXC motif regulates the catalytic efficiency of Glutaredoxin 3

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## **Supporting Information**

#### **Materials and Methods**

Buffers for kinetic measurements were prepared using de-ionized water.  $KH_2PO_4$  and  $K_2HPO_4$  were purchased from Fisher Biotech. Recombinant *E. coli* Trx1 was purchased from Promega Corporation (Madison, WI).

All Boc-amino acids were obtained from Midwest Biotech (Fishers, IN), with the following side chain protecting groups: Arg(Tos), Asp(OcHxl), Asn(Xan), Cys(MeBzl) Cys(Acm), Glu(OcHxl), Ser(Bzl), Thr(Bzl), Tyr(Br-Z), Lys(2ClZ), His(Bom) (OcHxl = cyclohexyl; Bzl = benzyl; MeBzl = 4-methylbenzyl; 2ClZ = 2-chlorobenzyloxycarbonyl; Br-Z = 2-Bromobenzyloxycarbonyl; Bom = benzyloxymethyl; Xan = N-Xanthyl; Acm = acetamidomethyl). 1H-Benzotriazolium-1-[bis(dimethylamino)methylene]-5chloro,hexafluorophosphate(1-),3-oxide (HCTU), S-Trityl-\beta-mercaptopropionic acid-Leu-OCH<sub>2</sub>-Pam (TMPAL-Pam) resin, were obtained from Peptides International (Louisville, KY). All solvents; HPLC-grade, N,N-dimethylformamide (DMF), dichloromethane, and acetonitrile (ACN), were purchased from Fisher. Trifluoroacetic acid (TFA) was obtained from Halocabon Products (River Edge, NJ). Anhydrous HF was purchased from Matheson Gas (Cucamonga, CA). Anisole was purchased from Sigma-Aldrich (St. Louis, MO). N,Ndiisopropylethyl amine (DIEA), and Boc-Lys(2ClZ)-OCH<sub>2</sub>-Pam resin from Applied Biosystems (Foster, CA). All other chemicals were obtained from Fisher or Sigma-Aldrich, Inc.

#### High Performance Liquid Chromatography (HPLC)

Analytical reversed-phase HPLC was performed on a Hewlett-Packard HPLC 1050 with 214 nm UV detection using Vydac C<sub>18</sub> column (5  $\mu$ m, 0.46 × 15 cm) or Varian system using a Phenomenex C<sub>18</sub> column (Jupiter 5  $\mu$ m, 300 Å, 150 × 4.6 mm) or C<sub>4</sub> (Jupiter 10  $\mu$ m, 90 Å, 150 × 4.6 mm). Semi-preparative reversed-phase HPLC was performed on a Gilson HPLC system using a Vydac C<sub>18</sub> column (10  $\mu$ m, 1.0 × 25 cm). Preparative reversed-phase HPLC was performed on Waters HPLC system using Vydac C<sub>18</sub> column (10  $\mu$ m, 2.5 × 25 cm). Linear gradients of acetonitrile in water with 0.1% TFA were used for all systems to elute bound peptides. The flow rates were 1 mL/min (analytical), 5 mL/min (Semi-preparative), and 30 mL/min (preparative). Buffer A is MilliQ water containing 0.1% TFA; buffer B is acetonitrile with 9.9% water and 0.1% TFA.

#### **Mass Spectrometry**

Electrospray ionization MS was performed on an API-III triple quadruple mass spectrometer (Sciex, Thornhill, ON, Canada). Peptide masses were calculated from the experimental mass to charge (m/z) ratios from all of the observed multiply charged species of a peptide by using MacSpec software (Sciex). Theoretical masses and the  $\varepsilon_{280nm}$  values of peptides and proteins were calculated by using *Sherpa*<sub>Lie</sub><sup>4.0</sup> for Mac.

#### **Fluorescence Spectroscopy**

Fluorescence measurements were made by using Fluoromax 2 fluorimeter (FluoroMax®-2; Jobin Yvon SPEX Instruments S.A., Inc., NJ, USA) equipped with a RK208P detector. Trx fluorescence was detected at the emission spectra of 345 nm, and excitation of 295 nm (excitation and emission slits 5 nm). All fluorescence experiments were preformed in triple for each analog in quartz cuvettes with optical paths of 5 nm x 1 nm.



**Figure S1**. A. NCL between Grx3(1-37)(Lys8Nva)-MPAL thioester peptide and Grx3(C38-82) peptide as followed by analytical HPLC (C-18 reversed-phase column, 20-55% acetonitrile over 25 mins, monitored at 214 nm), and ESI-MS. B. The expected mass of 9193 Da observed by ESI-MS reflects formation of the desired protein Grx3(Lys8Nva-A38C) with both Cys 11 and 14 protected with Acm.



**Figure S2**. ESI-MS of the ligated product Grx3(1-82)(Lys8Nva-A38C) protected at Cys11 and 14 with Acm after alkylation with iodoacetaminde gave the mass of the desired product (9250 Da).



**Figure S3**. ESI-MS of removal of two Acm protecting groups from Cys 11 and 14 in Grx3(1-82)(Lys8Nva-A38X) with  $I_2$ /MeOH. The observed mass of 9105 Da reflects the formation of the desired oxidized Grx3(1-82)(Lys8Nva-A38X) analog. X = (S-CH<sub>2</sub>CONH<sub>2</sub>)Cys.

### **Rate equations**



$$\begin{bmatrix} [C]_{0} = [D]_{0} = 0 \\ [A]_{0} = [B]_{0} \\ [A]_{t} = [A]_{0} - [C]_{t} \end{bmatrix} \\ \begin{bmatrix} A]_{t} = [A]_{0} - [C]_{t} \end{bmatrix} \\ \frac{d[C]_{t}}{dt} = k_{1}[A]_{t}[B]_{t} - k_{-1}[C]_{t}[D]_{t} \\ \frac{d[C]_{t}}{dt} = k_{1}[A]_{t}^{2} - k_{-1}[C]_{t}^{2} \\ \frac{d[C]_{t}}{dt} = k_{1}([A]_{0} - [C]_{t})^{2} - k_{-1}[C]_{t}^{2} \\ \frac{d[C]_{t}}{dt} = k_{1}([A]_{0}^{2} - 2[A]_{0}[C]_{t} + [C]_{t}^{2}) - k_{-1}[C]_{t}^{2} \\ \frac{d[C]_{t}}{dt} = k_{1}(A]_{0}^{2} - 2[A]_{0}[C]_{t} + (k_{1} - k_{-1})[C]_{t}^{2} \\ \frac{d[C]_{t}}{dt} = k_{1}[A]_{0}^{2} - 2k_{1}[A]_{0}[C]_{t} + (k_{1} - k_{-1})[C]_{t}^{2} \\ \frac{d[C]_{t}}{dt} + 2k_{1}[A]_{0}[C]_{t} + (k_{-1} - k_{1})[C]_{t}^{2} = k_{1}[A]_{0}^{2} \\ Therefore \qquad [C]_{t} = \frac{[A]_{0}(e^{2(Ab}\sqrt{k_{b}k_{-1}\cdot t} - 1)}{(e^{2(Ab}\sqrt{k_{b}k_{-1}\cdot t} - 1) + (\sqrt{\frac{k_{-1}}{k_{1}}}e^{2(Ab}\sqrt{k_{b}k_{-1}\cdot t} + 1)} \\ \begin{bmatrix} F_{0} = K_{A} \cdot [A]_{0} \\ F_{\infty} = K_{C} \cdot [C]_{\infty} \end{bmatrix} \\ F_{t} = [A]_{t} \cdot K_{A} + [C]_{t} \cdot K_{C} \\ F_{t} = [A]_{t} - [C]_{t} \cdot (K_{C} - K_{A}) \\ F_{0} - F_{t} = [C]_{t} \cdot (K_{A} - K_{C}) \\ 1 - \frac{F_{t}}{F_{0}} = \frac{[C]_{t} \cdot (K_{A} - K_{C})}{K_{A} \cdot [A]_{0}} \cdot \frac{[A]_{0}(e^{2(Ab}\sqrt{k_{b}k_{-1}\cdot t} - 1)}{(e^{2(Ab}\sqrt{k_{b}k_{-1}\cdot t} - 1) + (\sqrt{\frac{k_{-1}}{k_{1}}}e^{2(Ab}\sqrt{k_{b}k_{-1}\cdot t} + 1)} \\ \end{bmatrix}$$

And hence, the rate equation measured by Fluorescence as a function of time  $(F_t)$  is:

$$1 - \frac{F_t}{F_0} = (1 - \frac{K_C}{K_A}) \cdot \frac{(e^{2 \cdot [A]_0 \sqrt{k_1 k_{-1}} \cdot t} - 1)}{(e^{2 \cdot [A]_0 \sqrt{k_1 k_{-1}} \cdot t} - 1) + (\sqrt{\frac{k_{-1}}{k_1}} e^{2 \cdot [A]_0 \sqrt{k_1 k_{-1}} \cdot t} + 1)}$$

Where  $F_0$  is the fluorescence at time zero (the fluorescence of reduced Trx), and  $K_A$  and  $K_C$  are constants for fluorescence of A (Red. Trx) and C (Ox. Trx), respectively, and the  $K_C/K_A$  is ~0.18 analogous to the value reported by Holmgren.<sup>1</sup>

<sup>&</sup>lt;sup>1</sup> a) A. Holmgren, J. Biol. Chem., 1972, **247**, 1992-1998. b) A. Holmgren, Annu. Rev. Biochem, 1985, **54**, 237-271.