

SUPPORTING INFORMATION

Mechanism of Glutaredoxin – ISU [2Fe-2S] Cluster Exchange

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Experimental Methods

Expression, Purification, Reconstitution and Characterization of Recombinant human Grx2.

A construct of human Grx2 (comprising residues 56-161) with a tobacco etch virus cleavable N-terminal His₆ tag in expression vector pNic-Bsa4 was kindly provided by Drs. Kavanagh, Muller-Knapp and Oppermann. Plasmids were transformed into BL21-CodonPlus (DE3)-RIL competent cells for expression. Protein expression was induced by the addition of 1mM IPTG to the cell culture grown at 37 C in Terrific Broth to an OD = 3.4 and incubated at 25 C overnight before centrifuging to harvest cells. Cell pellets were then resuspended in 20 mM Tris, 500 mM NaCl, 5 mM imidazole, pH = 8.0 and lysed by sonication. Cell debris was removed by centrifugation at 4 C at 15,000 rpm for 30 min. The supernatant was then loaded onto a Ni-NTA column and eluted by standard methods, and further purified by gel filtration chromatography using a Superose12 column equilibrated with 50 mM Hepes, 100 mM NaCl, pH = 7.5. Eluted proteins were analyzed by SDS-PAGE and concentrated by Amicon ultrafiltration.

In vitro reconstitution of Grx2 was performed as follows: 200 μM apo human Grx2 was incubated with 2 μM *Tm* Nifs and 5 mM DTT, argon-purged for 30 min, and then made up to 0.6 mM in Fe²⁺ and 0.6 mM in L-cysteine. After incubation for 1 h, the reaction mixture was concentrated and passed through a Sephadex G-25 desalting column to remove excess Fe²⁺, S²⁻ and L-cysteine, and the UV-vis spectrum obtained for both apo and holo human Grx2.

Expression, Purification and Reconstitution of Native and Substituted Human ISU.

Apo and holo native and D46A derivative of human ISU were isolated and purified as previously described (*Biochemistry*, 2002, **41**, 8876-8885).

Glutaredoxin Assay (HED assay).

A fresh mixture of 0.7 mM β-hydroxyethyl disulfide (HED), 0.2 mM EDTA, 0.1 mg/mL BSA, 250 μM NADPH, 1 unit glutathione reductase, 1 mM GSH in 100 μL 50mM Hepes, 100 mM NaCl, pH=7.5 was incubated for 5 min. A 2 μL volume of either purified apo glutaredoxin or cluster transfer reaction solution was then added to the assay mixture and the change of absorbance was monitored at 340 nm for 1 min.

ISU-Grx2 Interaction Study by Isothermal Titration Calorimetry.

ITC measurements of human ISU binding to human Grx2 were carried out at 25 C on a MicroCal ultrasensitive titration calorimeter. A solution of 40 μ M apo native human ISU, and 1 mM TCEP in 50 mM Hepes pH 7.5 was rigorously degassed and loaded into the calorimeter cell. A 500 μ M solution of monomeric Grx2 in the same buffer was titrated into the sample in 10 μ L aliquots over a period of 24 s at 6 min intervals. The titration was repeated under the same conditions in the presence of 2 mM GSH in both the cell and the syringe, as well as in the presence of 2 mM GSSG without TCEP. Data were collected automatically and subsequently analyzed with Origin Software package (version 7.0) provided by MicroCal. Control experiments, following titration of titrant into buffer, or buffer into titrant were also performed and used to correct for background heat changes.

Cluster Transfer Reaction.

Components were mixed anaerobically from stocks to yield a final solution of either 50 μ M holo wild type human ISU, 15 μ M monomeric human Grx2, 1mM GSH and 5mM DTT; or 100 μ M apo D37A ISU, 10 μ M dimeric human Grx2, 1 mM GSH and 5 mM DTT, in 50 mM Hepes, 100 mM NaCl, pH = 7.5. At 10 min intervals, 2 μ L of the reaction mixture was withdrawn and used in the glutaredoxin HED assay. The slope of the absorbance change at 340 nm was plotted against reaction time. A control reaction was performed under the same conditions, with the exception that the control reaction mixture did not contain glutathione for the cluster transfer reaction from ISU to Grx2. A control for the cluster transfer reaction from Grx2 to ISU was conducted under similar conditions, but in the absence of ISU.

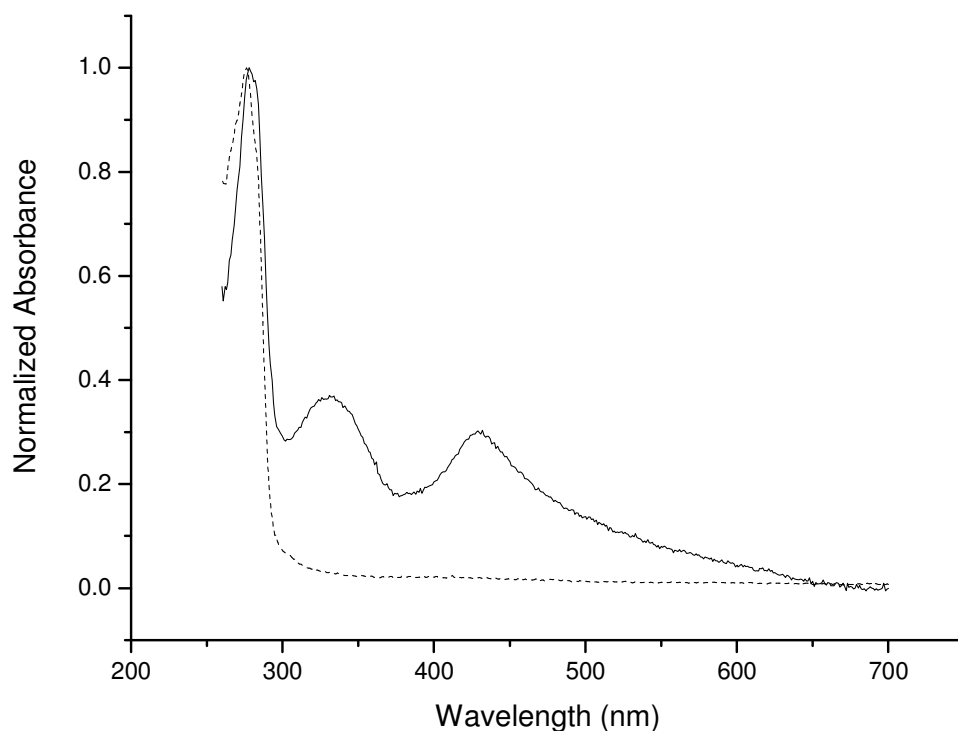


Figure S1. UV-vis spectra of monomeric apo Grx2 (dotted) and holo Grx2 dimer (solid) following reconstitution. Spectra are normalized to the 280 nm absorbance.

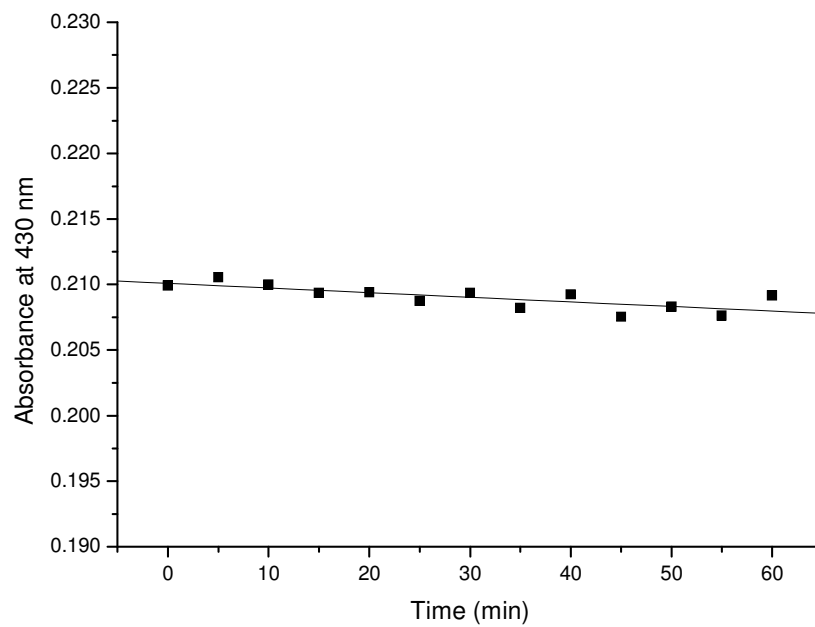


Figure S2. The Grx2-bound cluster is relatively stable in the presence of GSH. The plot shows the change in absorbance at 430 nm for reconstituted Grx2 in the presence of GSH over a period of 60 min.

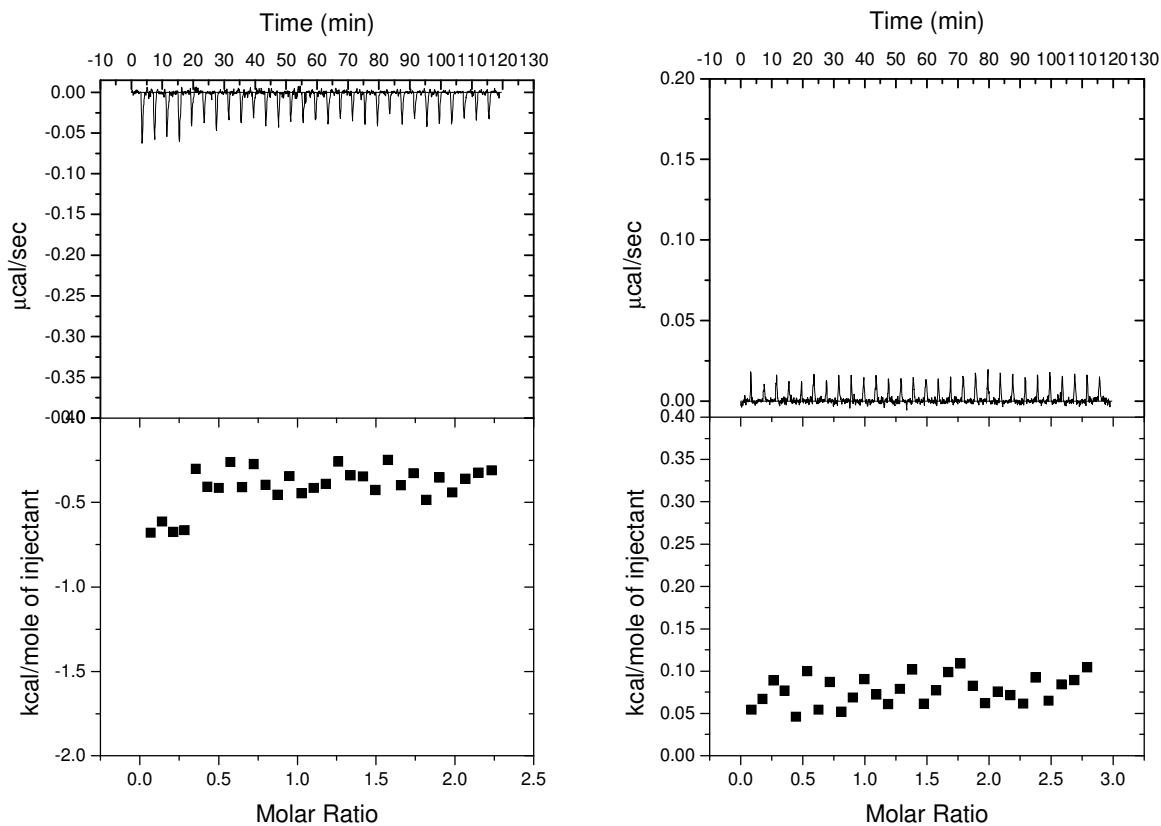


Figure S3. ITC study of the interaction between ISU and Grx2. (Left) no binding detected between apo native ISU and monomeric apo Grx2 in the presence of TCEP, but absence of GSSG; and (Right) no binding detected between apo native ISU and monomeric apo Grx2 in the presence of TCEP and GSH, but absence of GSSG.

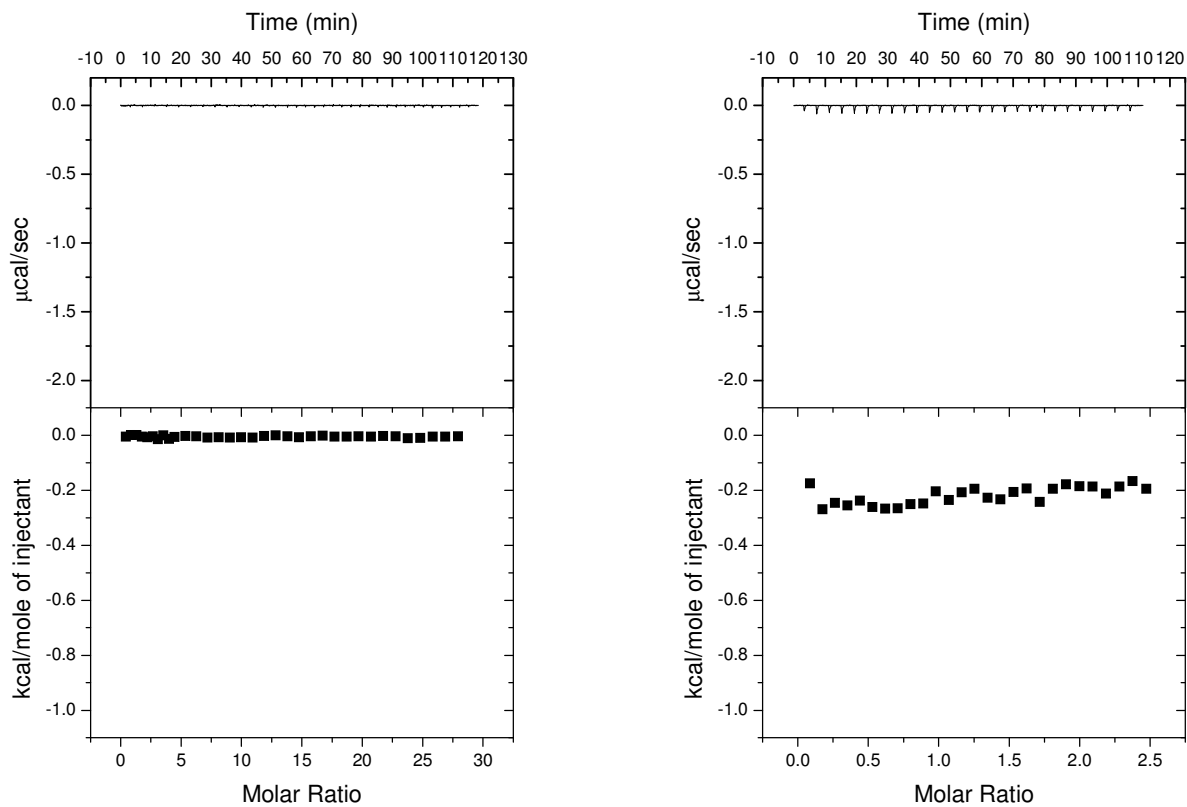


Figure S4. No binding was detected by ITC when GSSG was titrated into native ISU (left panel) or apo Grx2 (right panel), respectively.

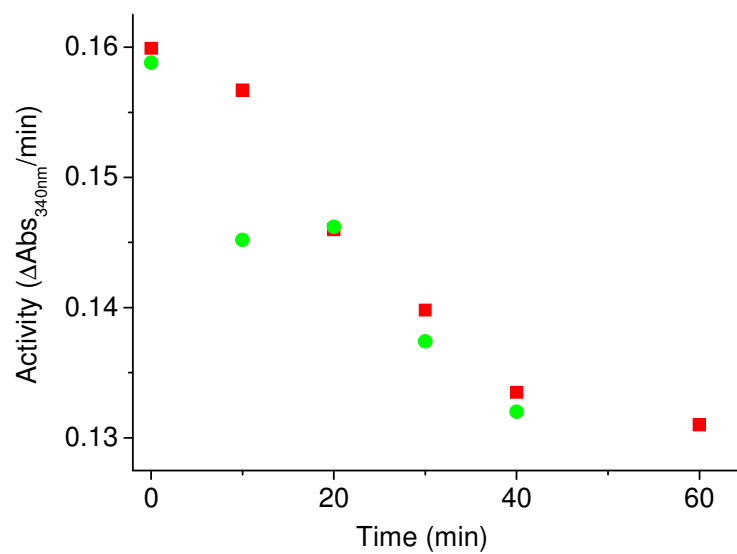


Figure S5. A 25 μM solution of Grx2 was incubated with 100 μM TCEP for 10 min, and then holo ISU (to 50 μM), and either 2mM GSH or 1 mM GSH/0.5 mM GSSG was added. The exchange reaction was monitored by use of the HED assay. Red: 1mM GSH + 0.5mM GSSH, Green: 2mM GSH.