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Oxidative Disassembly of the [2Fe-2S] Cluster of Human Grx2 and Redox Regulation in the Mitochondria[†]

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ABSTRACT: Mitochondrial Grx2 is a new member of the thioredoxin superfamily that has been found to bind a [2Fe-2S] cluster in a novel coordination motif at the interface of a homodimer, where cluster binding occurs via a catalytic cysteine residue and a molecule of GSH (per monomer). The $(Grx2)_2$−[2Fe-2S] dimer is thought to undergo cluster destruction and monomerization in a redox-induced pathway of activation. In this report, we make use of protein film voltammetry (PFV) as a method to probe the stability of the Grx2−[2Fe-2S] cluster, using oxidative poises of varying potential and duration to probe the thermodynamic and kinetic stability of the cluster's electrochemical response. We find that the cluster signal is stable at positive potentials up to 0.5 V but that cluster destruction occurs readily when oxidative pulses in excess of this value are applied.

To prevent intracellular damage resulting from reactive oxygen species (ROS), cells and organelles have evolved elaborate antioxidant defense mechanisms that include the glutaredoxin (Grx) system consisting of Grx, glutathione (GSH), and glutathione reductase (*1*, *2*). Grxs are small proteins that usually reduce protein disulfide bonds or protein−glutathione mixed disulfide bonds (*3*, *4*). Grx receives its reducing equivalents from GSH, a cellular thiol buffer, which in turn is reduced by NADPH and glutathione reductase (*3*, *5*). Human cells contain two dithiol Grxs: a well-studied cytosolic Grx1 (*4*) and a recently identified mitochondrial Grx2 (*6*, *7*). Mitochondrial Grx2 binds a $[2Fe\text{-}2S]^{2+}$ cluster by the catalytic cysteines of two monomer subunits and the cysteines of two GSH molecules (Figure 1) (*8*, *9*, *12*), and plant analogues with similar [2Fe-2S] cluster binding capability have been observed recently (*10*, *11*). The role of Grx2 FeS cluster binding and dimerization has been attributed to a redox sensing function: the dimeric form of the protein is maintained by the redox homeostasis of the mitochondria (*12*), while oxidative stress conditions are marked by the generation of uncontrolled ROS and the concomitant degradation of the $[2Fe\text{-}2S]^{2+}$ cluster through an unknown mechanism that monomerizes active Grx2. Mechanistically, Grx2 monomerization and/or cluster destruction could occur either through a shift in the GSH/GSSG ratio (as GSH is a direct ligand to the FeS cluster) or through redox chemistry occurring via outer-sphere electron transfer from ROS or other biological oxidants. Here, we have probed the latter of these possibilities by applying protein film voltammetry (PFV) (*13*) in a study of the stability of the [2Fe-2S] cluster of the $(Grx2)_2$−[2Fe-2S] dimer. PFV is used to define the thermodynamic limits of the [2Fe-2S] cluster stability in Grx2, as mediated by pure redox reactions.

We have immobilized the $(Grx2)_2$−[2Fe-2S] dimer on an electrode surface under anaerobic conditions and conducted cyclic voltammetry, which reveals an intense response due to the [2Fe-2S] cluster centered at approximately 0 V (Figure 2). We have found that protein films are stable for 1 h under these conditions at room temperature, and though the voltammetric response [as observed following the subtraction of the background current (Figure 2A, inset)] is slightly asymmetrical, the linear dependence of peak current upon scan rate (Figure 2B) indicates the protein behaves as an adsorbed species.

To probe the stability of the FeS cluster, we have investigated the PFV response as a function of potential, employing oxidative pulses of varying duration and magnitude (*14*). Figure 3 illustrates the experimental strategy: initial CV data were collected to establish the baseline Grx2 signal intensity, and then a fixed potential pulse of set reduction potential (an "oxidative poise") was applied for a variable amount of time (from 10 to 300 s). Immediately following the pulse, CV data were recorded to determine the impact of the poise upon the magnitude of the Grx2 voltammogram. A range of applied potential poise was assessed systematically in this manner, and through the poising potential range of 0−0.5 V, the intensity of the Grx2 voltammetry was not perturbed, indicating that the Grx2 cluster was oxidatively stable over this entire range. However, when the poising potential was increased from 0.5 to 0.6 V, the peak current was found to become smaller.

PFV was also used to probe the impact of the duration of the oxidative pulses used to disrupt the Grx2−[2Fe-2S] cluster. Figure 4A shows baseline-subtracted data for the immediate PFV probe response of Grx2 after treatment with various poise potentials of 0.5, 0.6, and 0.7 V (black, red, and blue, respectively) applied for durations of 0

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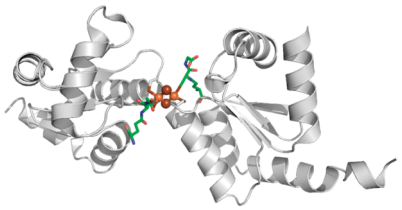

FIGURE 1: Grx2 dimer bearing a [2Fe-2S] cluster (spheres), featuring ligation by a protein-bound Cys and an additional molecule of GSH (sticks) per monomer (*9*). This figure was prepared from Protein Data Bank entry 2HT9 using Pymol (DeLano Scientific).

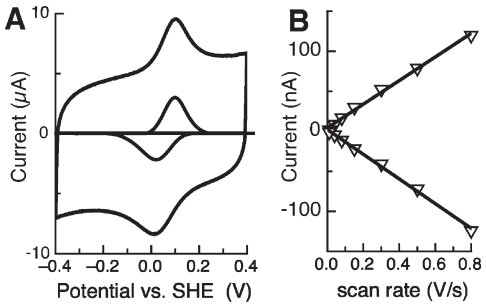

FIGURE 2: (A) PFV response of cluster-bound Grx2 upon 11-mercaptoundecanoic acid-modified gold electrode as both raw and baseline-subtracted data. Data were collected at pH 7.0, 10 °C, and 150 mV/s (ν). (B) Dependence of peak height (current) upon scan rate (ν), demonstrating a linear response, indicating that the PFV signal is due to an adsorbed species.

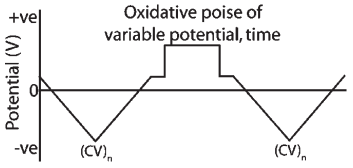

FIGURE 3: Sequence of stages in a poising-coupled CV experiment. CV is collected before and after an oxidative poise of precise potential and duration is applied.

(no pulse), 50, and 100 s. Figure 4A demonstrates that in the three different experiments, the Grx2 cluster signal is nearly identical initially, but then decay occurs as a function of both poise potential and time. Additionally, the peak height for the oxidative and reductive half-scans was used to represent the overall magnitude of the intact Grx2−[2Fe-2S] cluster, and Figure 4B illustrates how variable oxidative pulse duration impacts the Grx2 electrochemical signal. When the oxidative poise equals 0.5 V, the peak currents remain unaffected essentially. However, the cluster signal attenuates with time following the application of a 0.6 V pulse, while a 0.7 V pulse results in complete signal loss within 120 s. The resulting values of k_{obs} for cluster decay depend upon the potential of the poise: as the poise increases from 0.6 to 0.7 V, k_{obs} increases from 25 to 30 ms^{-1}. As a further control, blank electrodes (no Grx2) were subjected to identical pulsing experiments, and the electrode surface was found to be stable throughout the treatment. Thus, the Grx2 dimer bearing a [2Fe-2S] cluster appears to be unstable to pure redox reactions at potentials greater than 0.5 V, though the cause of this instability is not clear at this time.

Ideally, the loss of cluster should result in the appearance of a Grx monomer dithiol/disulfide redox signal in addi-

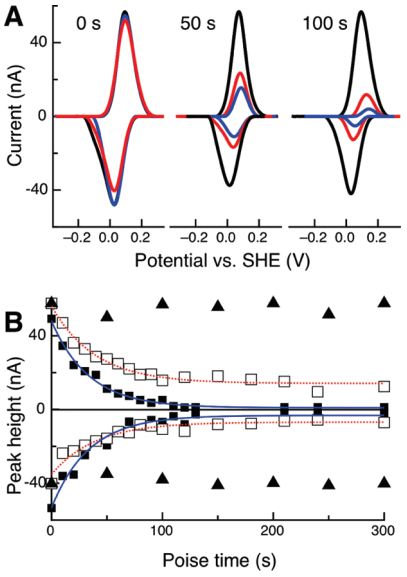

FIGURE 4: (A) Baseline-subtracted data corresponding to an oxidative poise-coupled CV experiment at 0, 50, and 100 s. The individual protein films were preconditioned at precise oxidative potentials of 0.5 V (black line), 0.6 V (red line), and 0.7 V (blue line) for a fixed duration of time, prior to collection of CV. (B) Time dependence of cluster disassembly. Oxidative and reductive peak currents are plotted as a function of time following the application of an oxidative pulse for a fixed duration and are fit to a first-order process (Supporting Information). The individual protein films were exposed to 0.5 (▲), 0.6 (□), and 0.7 V (■) oxidative poise for fixed time intervals, after which CV was immediately collected.

tion to the loss of the cluster redox couple. However, no new signal was observed using gold-based electrodes as in Figures 2 and 4. This is not entirely surprising. The Grx disulfide couple should be low in potential [−220 mV (*15*)], and the modified gold electrode used here is not suitable for investigating low-potential signals, generally. However, a stable monomer signal representing the liberated dithiol/disulfide could be obtained on a pyrolytic edge graphite electrode, a surface that is amenable to low reduction potentials (*16*). The Grx2 monomer reduction potential (E_m) was found to be −230 mV (Figure S1), in good agreement with the literature (*15*).

If the cluster were truly destroyed in the experiments described above, then electrochemical cell solutions should contain "FeS" and GSH following the PFV experiments. This hypothesis was verified qualitatively by taking cell solutions following oxidative poising experiments like those in Figure 4 and demonstrating that Fe and GSH are free in the cell solution after cluster destruction (Figure S2). To test if following apparent PFV-induced cluster destruction the entire protein dimer has desorbed from the electrode in the course of the experiments, several electrochemical cell solutions were collected and concentrated, yet Grx2 could not be detected by either silver-stained SDS−PAGE analysis or Grx activity assays. (Notably, surface coverages of Grx2 are typically 10−15 pmol/cm^2, such that if the entire loss of the PFV response is due to protein desorption, the concentration of the resulting protein-containing solution should yield approximately 1.5 ng of protein, which is within the limit of detection using silver-stained SDS−PAGE.) As Fe and GSH were detected in cell solutions following apparent cluster destruction and free Grx2 monomer was not, we conclude

that in our PFV experiments there is little loss of the Grx2 dimer due to desorption and that the observed current loss in oxidatively poised PFV (Figure 4) is due to cluster disassembly. In this model, FeS and GSH are liberated into the cell solution following cluster destruction, and the Grx monomer remains adsorbed to the electrode.

To support further our model for PFV-induced cluster destruction in the $(Grx2)_2$−[2Fe-2S] dimer, we verified that the adsorbed $(Grx2)_2$−[2Fe-2S] dimer displays additional reactivity identical to that of the native protein. We probed the accessibility of the [2Fe-2S] cluster of the adsorbed $(Grx2)_2$−[2Fe-2S] dimer by taking protein films that were interrogated by PFV and then exposing the entire electrode surface to the exogenous oxidizing agent (GSSG), and the fate of the cluster was monitored electrochemically (Figure S3). We found that at the electrode, a steady loss of cluster signal was observed when a modified gold electrode with immobilized cluster-bearing Grx2 was rotated in an electrochemical cell solution in the presence of increasing amounts of GSSG in the bulk phase, as would be predicted on the basis of the reactivity of the cluster-bearing dimer in solution, monitored spectroscopically (Figure S4). Thus, while the $(Grx2)_2$−[2Fe-2S] dimer is immobilized at the electrode, the [2Fe-2S] cluster bound in the Grx2 dimer is accessible freely to solution, consistent with a model in which application of poising potentials mimics the oxidizing potential of outer-sphere redox reagents.

Our data indicate that the [2Fe-2S] cluster of the Grx2 dimer is stable at the electrode to nominal one-electron redox chemistry, as the $[2Fe\text{-}2S]^{2+/+}$ couple is interrogated in Figures 2 and 4, yet the same protein appears to be sensitive to oxidizing reagents of sufficiently high potential. The PFV experiment enables the interrogation of the interplay between electron transfer chemistry and cluster stability; however, the electrode is a poor surrogate for specifically mimicking a change in the GSH/GSSG ratio, as GSH itself is found within the Grx2−[2Fe-2S] cluster. Indeed, the fact that we can observe a stable voltammetric signal for the cluster unless a high oxidative pulse is applied indicates that the $(Grx2)_2$−[2Fe2S] dimer will be stable in the mitochondria in the absence of specific interactions with glutathione, or strong oxidants. The following model emerges from our results: cluster-bound Grx dimer may remain intact below 0.5 V poise potentials when experiencing weakly to moderately oxidizing conditions. However, under oxidative stress conditions imitated by poise potentials greater than 0.5 V, the cluster is lost and enzymatically competent Grx2 and GSH are released. Consistent with our data is the fact that H_2O_2 [one-electron reduction couple at 0.38 V (*19*)] cannot induce disintegration of the cluster (*12*), while cluster destruction could occur by the direct action of a stronger oxidizing reagent such as the ROS superoxide, O_2^- [0.89 V (*19*)]. In fact, Grx2 overexpression attenuates apoptosis induced by doxorubicin (*12*), which generates O_2^- in its target cells (*20*). In agreement with this model, Grx2 is designed to function primarily under oxidative conditions (*17*), including the ability to accept electrons from thioredoxin reductase (*18*), a requirement when GSH becomes depleted under oxidizing conditions.

We have presented data directly supporting the proposal that the [2Fe-2S] cluster form of human mitochondrial Grx2 can behave as a redox sensor, which is sensitive to the potential applied by an electrode as an outer-sphere oxidant. Currently, it appears that the $(Grx2)_2$−[2Fe-2S] dimer can be activated by both an inner-sphere mechanism (direct reaction with GSSG) and outer-sphere processes. Thus, we believe that that PFV coupled with poising pretreatments provides for quantitative assessments of the thermodynamic limits of in vivo oxidative stress due to outer-sphere oxidation reactions, thereby providing an in vitro tool for analyzing the impact of oxidizing conditions on redox-regulated proteins. To the best of our knowledge, this is the first time PFV has been applied to the redox-triggered disassembly of a [2Fe-2S] cluster.

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SUPPORTING INFORMATION AVAILABLE

Experimental procedures and Figures S1−S5. This material is available free of charge via the Internet at http://pubs.acs.org.

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