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Light-induced release of nitric oxide from the nitric oxide-bound CDGSH-type [2Fe–2S] clusters in mitochondrial protein Miner2

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ABSTRACT

Human mitochondrial matrix protein Miner2 hosts two [2Fe–2S] clusters via two CDGSH (Cys-Asp-Gly-Ser-His) motifs. Unlike other iron-sulfur clusters in proteins, the reduced CDGSH-type [2Fe–2S] clusters in Miner2 are able to bind nitric oxide (NO) and form stable NO-bound [2Fe–2S] clusters without disruption of the clusters. Here we report that the NO-bound Miner2 [2Fe–2S] clusters can quickly release NO upon the visible light excitation. The UV–visible and Electron Paramagnetic Resonance (EPR) measurements show that the NO-bound Miner2 [2Fe–2S] clusters are converted to the reduced Miner2 [2Fe–2S] clusters upon the light excitation under anaerobic conditions, suggesting that NO binding in the reduced Miner2 [2Fe–2S] clusters is reversible. Additional studies reveal that binding of NO effectively inhibits the redox transition of the Miner2 [2Fe–2S] clusters, indicating that NO may modulate the physiological activity of Miner2 in mitochondria by directly binding to the CDGSH-type [2Fe–2S] clusters in the protein.

1. Introduction

Iron-sulfur proteins are involved in diverse physiological functions ranging from energy metabolism to DNA replication and repair [1]. Most iron-sulfur clusters in proteins are ligated via cysteine residues. However, other amino acid residues such as histidine, glutamine, serine, or arginine may also provide ligands for iron-sulfur clusters in proteins [2]. A new group of iron-sulfur proteins that contain the CDGSH (Cys-Asp-Gly-Ser-His) motifs have recently been identified [3]. Each CDGSH motif in the protein hosts a [2Fe–2S] cluster via a unique ligand arrangement of three cysteine and one histidine residues. In human mitochondria, there are three CDGSH-type iron-sulfur proteins [3,4]: mitoNEET [5], a mitoNEET-related protein 1 (Miner1 or NAF-1) [6], and a mitoNEET-related protein 2 (Miner2) [7,8]. MitoNEET was initially identified as a target of the type II diabetes drug pioglitazone [5]. Miner1 and mitoNEET are homologues with 54% identity and 76% similarity. Mutations of Miner1 have been attributed to causing type II Wolfram Syndrome [9,10]. Both mitoNEET and Miner1 contain a transmembrane alpha helix in the N-terminus and a CDGSH motif in the C-terminal domain that hosts a [2Fe–2S] cluster [6,11–13]. While mitoNEET is a mitochondrial outer membrane [5], Miner1 is localized in the mitochondrial outer membrane and the endoplasmic reticulum membrane [14]. Increasing evidence suggests that mitoNEET and Miner1 have a crucial regulatory role in cellular redox state, iron homeostasis, and production of reactive oxygen species in mitochondria [15,16]. Interestingly, unlike mitoNEET and Miner1, Miner2 is a soluble mitochondrial matrix protein and hosts two [2Fe–2S] clusters via two CDGSH motifs [7,8]. While it has been proposed that Miner2 could be involved in iron-sulfur cluster biogenesis in mitochondria [8], the physiological function of Miner2 remains largely elusive.

Iron-sulfur clusters in proteins are among the primary targets of nitric oxide (NO), as NO has a specific reactivity with ferrous iron in proteins [17–20]. A number of iron-sulfur proteins have previously been characterized as NO sensors in cells [20]. It has been shown that iron-sulfur clusters in proteins can be readily modified by NO forming the protein-bound dinitrosyl iron complexes [18,21,22], the thiolate-bridged diiron tetranitrosyl complexes [23], or the octa-nitrosyl clusters [24], depending on specific proteins and NO concentrations used in the experiments. Unexpectedly, we have found that the CDGSH-type [2Fe–2S] clusters in Miner2 are able to bind NO and form the stable NO-bound [2Fe–2S] clusters without disruption of the clusters [7]. In the present study, we report that the NO-bound Miner2 [2Fe–2S] clusters can readily release NO upon light excitation in the visible range. The UV–visible and Electron Paramagnetic Resonance (EPR) measurements show that the NO-bound Miner2 [2Fe–2S] clusters are converted to the reduced Miner2 [2Fe–2S] clusters after NO is released upon the light excitation, suggesting that NO binding in the reduced Miner2 [2Fe–2S] clusters is reversible. Furthermore, NO binding

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effectively inhibits the redox transition of the Miner2 [2Fe–2S] clusters. We propose that NO may modulate the function of Miner2 in mitochondria via directly binding to the CDGSH-type [2Fe–2S] clusters in the protein.

2. Materials and methods

2.1. Protein preparation

The gene encoding the human Miner2 (containing residues 34–127) with the N-terminal His tag was synthesized (Genscript co.) and cloned into pET28b + plasmid for protein expression in *E. coli* cells. The cloned DNA sequence was confirmed by direct sequencing (Eurofins MWG Operon). Overnight *E. coli* cells containing expression plasmid were diluted 1:100 in freshly prepared LB (Luria-Bertani) medium and incubated at 37 °C with aeration (250 rpm) for 3 h, followed by protein induction by adding Isopropyl β-D-1-thiogalactopyranoside (200 μM) overnight at 18 °C. The recombinant protein was purified as described previously [25]. The purity of purified proteins was greater than 95% as judged by electrophoresis analysis on a 15% polyacrylamide gel containing SDS followed by staining with Coomassie Blue. The NO-bound Miner2 [2Fe–2S] clusters were prepared by treating the *E. coli* cells expressing human Miner2 with NO gas (20 μM) or treating the reduced Miner2 [2Fe–2S] clusters with NO gas under anaerobic conditions as described previously [7]. Nitric oxide gas (Praxair Co.) was first passed through a soda-lima column to remove NO_2 and higher nitrogen oxides before being used to bubble pre-degassed water in a sealed 50-ml flask for 5 min. The concentration of nitric oxide in the NO-saturated solution was measured using a nitric oxide electrode (World Precision Instrument.) [26]. The UV–visible absorption spectra were recorded in a Beckman DU640 UV–visible spectrometer equipped with a temperature controller. The extinction coefficient of 11.88 $cm^{-1}mM^{-1}$ was used to determine the concentration of apo-Miner2.

2.2. Chemicals

NADH, isopropyl-β-D-1-thiogalactopyranoside, and kanamycin were purchased from Research Product International co. DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein) was purchased from ThermoFisher Scientific co. The diethylamine NONOate and Griess Reagent were purchased from Cayman Chemical co. FMN and other chemicals were purchased from Sigma co. The extinction coefficients of 6.2 $mM^{-1}cm^{-1}$ at 340 nm and 12.5 $mM^{-1}cm^{-1}$ at 445 nm were used for determining the concentrations of NADH and FMN, respectively [27]. $FMNH_2$ was prepared by using NADH and *E. coli* flavin reductase under anaerobic conditions as described in Ref. [28].

2.3. NO analyses

The NO-bound Miner2 [2Fe–2S] clusters were exposed to either room light (600 Lux) or a Cole-Parmer 41,720 series Illuminator A (400k Lux) which provides strong visible light source under aerobic or anaerobic conditions. The Cole-Parmer illuminator was equipped with an infrared filter that reduces the transmittance of heat to the sample. The intensity of the light excitation was measured using a Digital Lux Meter (LX1330B, Dr. Meter). Anaerobic conditions were achieved by purging a gas-sealed cuvette with pure Argon gas for 10 min in dark. DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein) was used to detect NO release. DAF-FM interacts with NO to form a benzotriazole product which has fluorescence (ThermoFisher Scientific co). The fluorescence spectra were taken upon excitation at 485 nm in a spectrofluorometer (FP-6300 Spectrofluorometer, Jasco Co.). The fluorescence intensity was also measured in a plate reader (Victor3 1420 Multilabel Counter, PerkinElmer Co.) using the emission wavelength at 535 nm and the excitation wavelength at 485 nm. Similar results were obtained from the spectrofluorometer and the plate reader. The NO-releasing reagent diethylamine NONOate (Cayman Chemicals co.) was used as the NO standard solution. A few crystals of diethylamine NONOate was first dissolved in buffer containing Tris (20 mM, pH 10.5), and the concentration of the NONOate was determined at 250 nm using an extinction coefficient of 6.5 $mM^{-1}cm^{-1}$. The NONOate was then transferred to buffer containing 20 mM Tris (pH 8.0) and 500 mM NaCl for the NO release under anaerobic conditions. Each NONOate molecule releases 1.5 equivalents of NO in buffer containing 20 mM Tris (pH 8.0) and 500 mM NaCl after incubation at 37 °C for 10 min (Cayman Chemicals co.).

2.4. EPR measurements

The X-band Electron Paramagnetic Resonance (EPR) spectra were recorded using a Bruker model ESR-300 spectrometer equipped with an Oxford Instruments 910 continuous flow cryostat. Routine EPR conditions: microwave frequency, 9.47 GHz; microwave power, 10.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; temperature, 20 K; receiver gain, 2×10^5.

2.5. Mass spectrometry measurements

For the mass spectrometry measurements, the protein samples were dissolved in 20 mM ammonium acetate (pH 8.0) buffer. The protein samples were either exposed to light excitation (400k lux) or left in dark at room temperature. Mass spectrometry experiments were carried out in the LSU mass spectrometry facility. Protein concentration was about 10 μM. All experiments were performed with an AmaZon speed electron transfer dissociation Ion Trap mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Sample solution was infused via an Apollo II electrospray ion source using a syringe pump at the flow rate of 3 μL/min. MS detection was performed in a full-scan mode in positive ionization enhanced resolution mode with the scan speed of 8100 *m/z*/s. The parameter settings for ESI-MS were as follows: capillary voltage, 4500 V; end plate offset, −500 V; nebulizer, 8 psi; dry gas, 4 L/min; dry gas temperature, 100 °C. MS data were acquired from 300 to 3000 with a target mass of 1200 *m/z* and trap drive level of 100%. The software used for data processing and de-convolution was Compass DataAnalysis (Bruker Daltonics, Billerica, MA, USA). The mass spectrometry data were analyzed using mMass software [29].

3. Results

3.1. The NO-bound Miner2 [2Fe–2S] clusters are light sensitive

Purified human Miner2 [2Fe–2S] clusters are in an oxidized state which have an absorption peak at 458 nm. When the Miner2 [2Fe–2S] clusters are reduced, the absorption peak at 458 nm is shifted to 420 nm [7]. In the previous studies, we have found that the reduced Miner2 [2Fe–2S] clusters, but not the oxidized ones, are able to bind nitric oxide (NO) and form the stable NO-bound Miner2 [2Fe–2S] clusters which have a new distinct absorption peak at 422 nm [7].

In the subsequent studies, we have noticed that when the NO-bound Miner2 [2Fe–2S] clusters were exposed to room light under aerobic conditions, the absorption peak at 422 nm of the NO-bound Miner2 [2Fe–2S] clusters was gradually shifted to the absorption peak at 458 nm of the oxidized Mienr2 [2Fe–2S] clusters (Fig. 1A). This observation let us to speculate that the NO-bound Miner2 [2Fe–2S] clusters could be light sensitive. To test the idea, the NO-bound Miner2 [2Fe–2S] clusters were subjected to a strong light source (400K Lux) which provides strong visible light under aerobic conditions. Fig. 1B shows that after the strong visible light excitation, the absorption peak at 422 nm of the NO-bound Miner2 [2Fe–2S] clusters was quickly changed to the absorption peak at 458 nm of the oxidized Miner2 [2Fe–2S] clusters. The light excitation for about 30 s was sufficient to fully convert the NO-bound Miner2 [2Fe–2S] clusters) to the oxidized

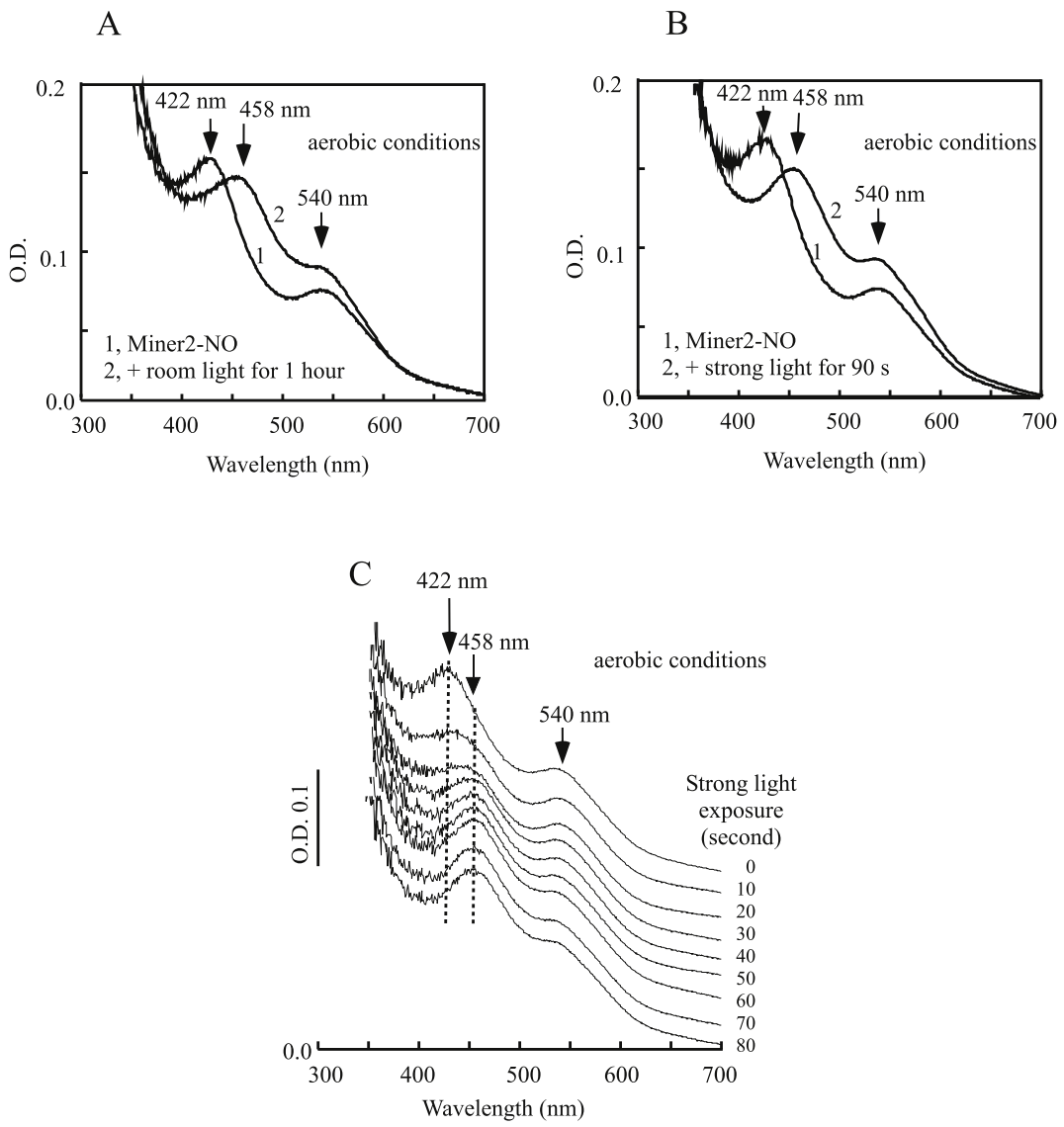

Fig. 1. The NO-bound Miner2 [2Fe–2S] clusters are light sensitive. A), effect of room light on the NO-bound Miner2 [2Fe–2S] clusters. Spectrum 1, the NO-bound Miner2 [2Fe–2S] clusters (10 μM) were incubated under dark for 1 h under aerobic conditions. Spectrum 2, the NO-bound Miner2 [2Fe–2S] clusters (10 μM) were incubated under room light for 1 h under aerobic conditions. **B)**, effect of the light excitation on the NO-bound Miner2 [2Fe–2S] clusters. Spectrum 1, the NO-bound Miner2 [2Fe–2S] clusters (10 μM) were incubated in dark for 90 s under aerobic conditions. Spectrum 2, the NO-bound Miner2 [2Fe–2S] clusters (10 μM) were exposed to the strong light excitation (400K Lux) for 90 s under aerobic conditions. **C)**, transition from the NO-bound Miner2 [2Fe–2S] clusters to the oxidized Miner2 [2Fe–2S] clusters upon the light excitation. The NO-bound Miner2 [2Fe–2S] clusters were exposed to the strong light excitation (400K Lux) for the indicated time. The absorption spectra were taken after each light exposure. Absorption peaks at 422 nm and 458 nm represent the NO-bound Miner2 [2Fe–2S] clusters and the oxidized Miner2 [2Fe–2S] clusters, respectively.

Miner2 [2Fe–2S] clusters under aerobic conditions (Fig. 1C). Thus, the NO-bound Miner2 [2Fe–2S] clusters are light sensitive, and the NO-bound Miner2 [2Fe–2S] clusters may be converted to the oxidized Miner2 [2Fe–2S] clusters by the light excitation under aerobic conditions.

3.2. The NO-bound Miner2 [2Fe–2S] clusters release NO upon light excitation

Previous studies have shown that when the NO-bound Miner2 [2Fe–2S] clusters were treated with Griess Reagent, nitrite was released from the NO-bound clusters due to denaturation of the protein [7]. Here, we used the Griess Reagent to explore whether the NO was actually removed from the NO-bound [2Fe–2S] clusters in Miner2 upon the light excitation. In the experiments, after the NO-bound Miner2 [2Fe–2S] clusters were exposed to the light excitation, the protein was re-purified by passing through a HiTrap Desalting column (GE Healthcare Life Sciences). Re-purified protein was then treated with Griess Reagents. As shown in Fig. 2A, the light excitation almost completely eliminated the acid-labile nitrite of the NO-bound [2Fe–2S] clusters in Miner2. On the other hand, the [2Fe–2S] clusters in Miner2 remained intact after the light excitation, suggesting that the NO-bound Miner2 [2Fe–2S] clusters only release NO upon the light excitation.

The NO-bound Miner2 [2Fe–2S] clusters were also subjected to the Electrospray Ionization (ESI) mass spectrometry analyses before and after the light excitation. As shown in Fig. 2B, the NO-bound Miner2 [2Fe–2S] clusters had a major mass peak at 12,889 before the light excitation. After the light excitation, the mass peak at 12,889 disappeared and was replaced with a new mass peak at 12,827, a mass decrease of 62 Da. The same mass peak at 12,827 was observed in the purified Miner2 [2Fe–2S] clusters which represents the sum of the Miner2 peptide (12,784) with a likely N-terminal acetylation (43) [30]

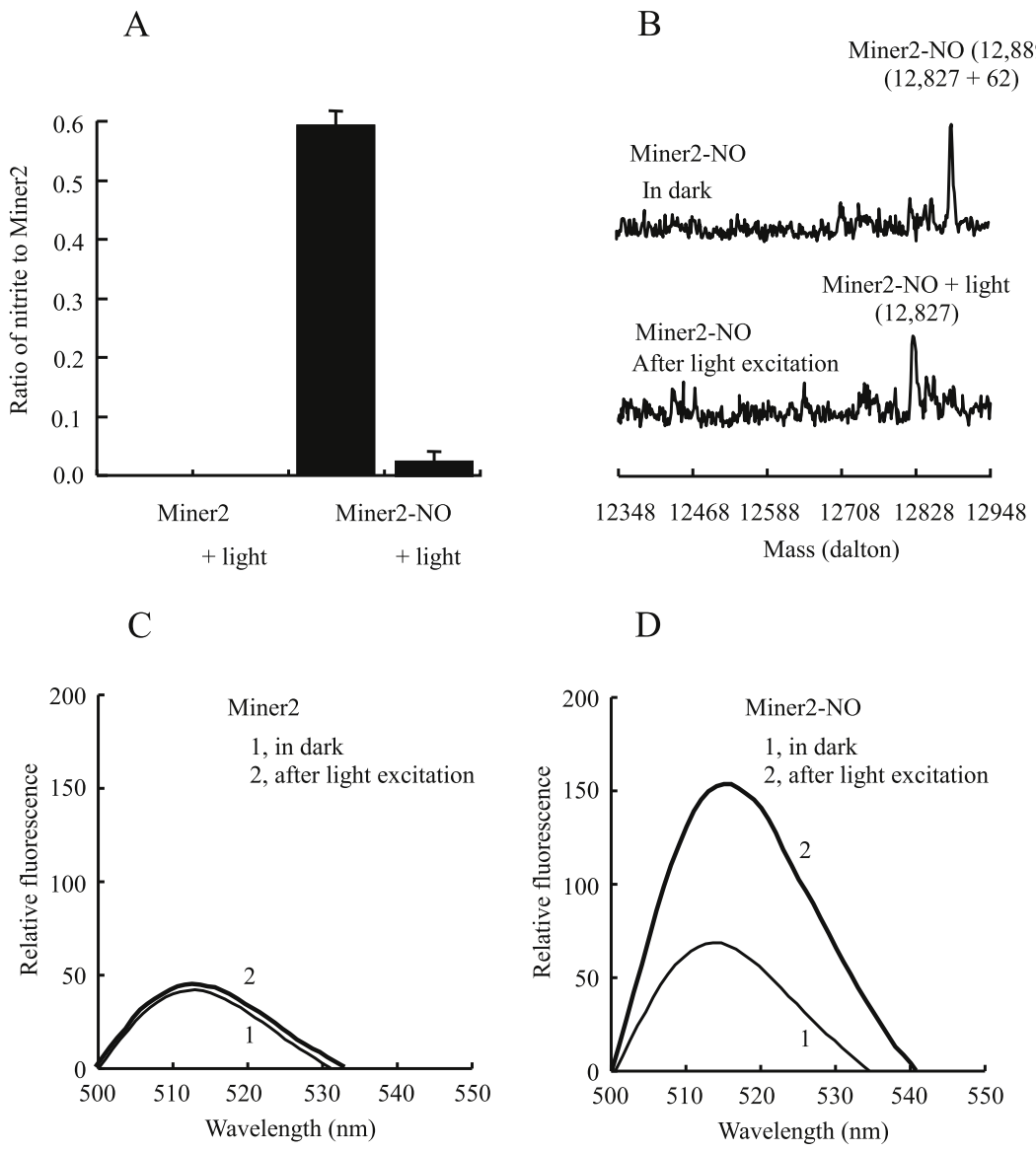

Fig. 2. NO is released from the NO-bound Miner2 [2Fe–2S] clusters upon light excitation. A), the NO-bound Miner2 [2Fe–2S] clusters release NO after the light excitation. The Miner2 [2Fe–2S] clusters or the NO-bound Miner2 [2Fe–2S] clusters (10 μM each) were either kept in dark or subjected to the light excitation (400K Lux) 60 s, followed by re-purification of the protein by passing through a High-Trap Desalting column. Re-purified proteins were then incubated with Griess Regents to determine the acid-labile nitrite. Sodium nitrite solution (1 mM) was used as a standard. Data were averages ± standard deviation from three experiments. **B),** mass spectra of the NO-bound Miner2 [2Fe–2S] clusters before and after the light excitation (400K Lux for 60 s). Spectrum 1, the NO-bound Miner2 [2Fe–2S] clusters. Spectrum 2, same as in spectrum 1 except after the light excitation. The mass spectra were deconvoluted using mMass software [29]. **C),** fluorescence spectra of DAF-FM incubated with the Miner2 [2Fe–2S] clusters under dark (spectrum 1) or after the light excitation (spectrum 2). The Miner2 [2Fe–2S] clusters (10 μM) were incubated with DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein) (10 μM) in dark for 2 min at room temperature under aerobic conditions. The samples were either kept in dark or exposed to the light excitation (400K Lux) for 60 s. The fluorescence spectra were taken using the excitation wavelength at 485 nm. **D),** fluorescence spectra of DAF-FM incubated with the NO-bound Miner2 [2Fe–2S] clusters under dark (spectrum 1) or after the light excitation (spectrum 2). Same as in C), except the NO-bound Miner2 [2Fe–2S] clusters (10 μM) were used.

and two [2Fe–2S] clusters (2 × 176). As each Miner2 protein hosts two [2Fe–2S] clusters and each [2Fe–2S] cluster can bind one NO [7], release of two NO molecules from the NO-bound Miner2 [2Fe–2S] clusters by the light excitation would result in the mass decrease by 62 Da. Thus, the mass spectrometry results suggested that the NO-bound Miner2 [2Fe–2S] clusters release two NO molecules upon the light excitation.

To further explore whether NO gas was released from the NO-bound Miner2 [2Fe–2S] clusters by the light excitation, we used the NO probe DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein) [31]. DAF-FM is essentially nonfluorescent until it reacts with NO to form a fluorescent benzotriazole (Thermo Fisher Scientific co). In the experiments, the Miner2 [2Fe–2S] clusters or the NO-bound Miner2 [2Fe–2S] clusters were incubated with DAF-FM under aerobic conditions, followed by the light excitation (400K Lux for 90 s). Fig. 2C shows that when the Miner2 [2Fe–2S] clusters were exposed to the light excitation, very little change of the fluorescence intensity of DAF-FM was observed. On the other hand, when the NO-bound Miner2 [2Fe–2S] clusters mixed with DAF-FM were exposed to the light excitation, the fluorescence intensity of DAF-FM was significantly increased (Fig. 2D). Using the diethylamine NONOate as the NO standard solution, we estimated that about 3.4 ± 0.2 μM NO was released from 10 μM of the NO-bound Miner2 [2Fe–2S] clusters after the light excitation.

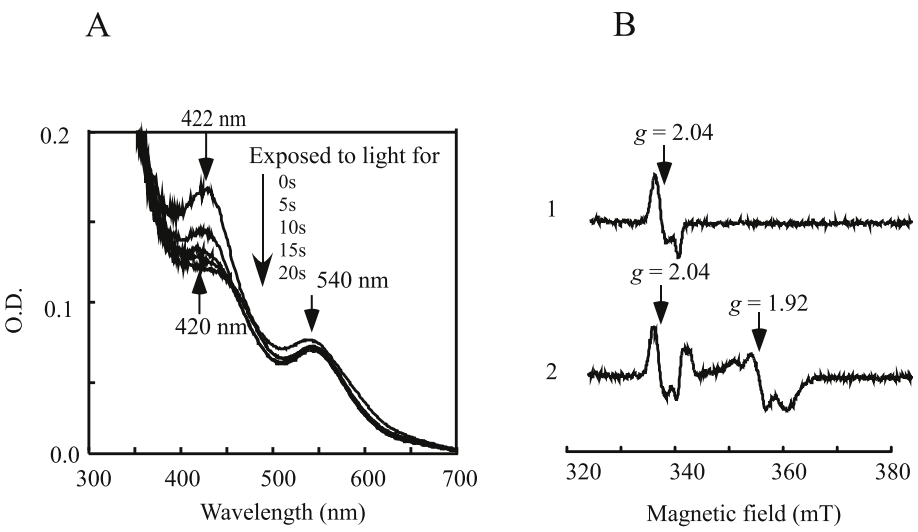

Fig. 3. Oxygen is not required for the light-induced NO release from the NO-bound Miner2 [2Fe–2S] clusters. A), UV–visible absorption spectra of the NO-bound Miner2 [2Fe–2S] clusters before and after the light excitation under anaerobic conditions. The NO-bound Miner2 [2Fe–2S] clusters (10 μM) were incubated under anaerobic conditions, followed by exposure to the light excitation (400K Lux) for 0, 5, 10, 15, and 20 s. Spectra were taken after each light excitation. **B)**, EPR spectra of the NO-bound Miner2 [2Fe–2S] clusters. Spectrum 1, the NO-bound Miner2 [2Fe–2S] clusters (10 μM). Spectrum 2, same as in spectrum 1 except after the light excitation (400K Lux for 60 s) under anaerobic conditions. The results are representatives from three independent experiments.

3.3. The NO-bound Miner2 [2Fe–2S] clusters are converted to the reduced Miner2 [2Fe–2S] clusters by the light excitation under anaerobic conditions

To determine whether oxygen is required for the light-induced NO release from the NO-bound Miner2 [2Fe–2S] clusters, we measured the UV–visible absorption spectrum of the NO-bound Miner2 [2Fe–2S] clusters before and after the light excitation under anaerobic conditions. Fig. 3A shows that after the light excitation, the absorption peak at 422 nm of the NO-bound Miner2 [2Fe–2S] clusters was shifted to the peak at 420 nm. Because the absorption peak at 420 nm reflects the reduced Miner2 [2Fe–2S] clusters [7], the results suggested that the NO-bound Miner2 [2Fe–2S] clusters are converted to the reduced Miner2 [2Fe–2S] clusters by the light excitation under anaerobic conditions.

In previous studies, we have shown that the reduced Miner2 [2Fe–2S] clusters have a distinct Electron Paramagnetic Resonance (EPR) signal at $g = 1.92$, and the NO-bound Miner2 [2Fe–2S] clusters are EPR-silent [7]. Here, we used EPR to examine whether the reduced Miner2 [2Fe–2S] clusters are actually formed when the NO-bound Miner2 [2Fe–2S] clusters are exposed to the light excitation under anaerobic conditions. Fig. 3B shows that the NO-bound Miner2 [2F–2S] clusters had an EPR signal at $g = 2.04$ which represented a small amount of dinitrosyl iron complex formed during the NO treatment of Miner2 [7]. When the NO-bound Miner2 [2Fe–2S] clusters were exposed to the light excitation under anaerobic conditions, a new EPR signal at $g = 1.92$ of the reduced Miner2 [2Fe–2S] clusters appeared (Fig. 3B). Integration of the EPR signal at $g = 1.92$ revealed that about 6.2 ± 0.5 μM of the reduced Miner2 [2Fe–2S] clusters were formed after 10 μM of the NO-bound Miner2 [2Fe–2S] clusters were exposed to the light excitation under anaerobic conditions. It should be pointed out that the EPR signal at $g = 2.04$ of the dinitrosyl iron complex was not changed upon the light excitation (Fig. 3B), indicating that dinitrosyl iron complex was not light sensitive. Taken together, the results established that the NO-bound Miner2 [2Fe–2S] clusters are converted to the reduced Miner2 [2Fe–2S] clusters by the light excitation under anaerobic conditions.

3.4. NO binding inhibits the redox transition of the Miner2 [2Fe–2S] clusters

Like the mitoNEET [2Fe–2S] clusters [28], the Miner2 [2Fe–2S] clusters can be reduced by the reduced flavin mononucleotide ($FMNH_2$) and oxidized by oxygen. Fig. 4A shows that the oxidized Miner2 [2Fe–2S] clusters were readily reduced by $FMNH_2$ under anaerobic conditions, and that the reduced Miner2 [2Fe–2S] clusters were re-oxidized after exposure to air, indicating that the Miner2 [2Fe–2S] clusters can undergo redox transition. In contrast, the NO-bound Miner2 [2Fe–2S] clusters could not be reduced by $FMNH_2$ or oxidized by oxygen, as the absorption peak at 422 nm of the NO-bound Miner2 [2Fe–2S] clusters remained the same upon addition of $FMNH_2$ or exposure to air (Fig. 4B). The results suggested that the NO-bound Miner2 [2Fe–2S] clusters are resistant to redox transition. When the NO-bound Miner2 [2Fe–2S] clusters were exposed to the light excitation, the NO-bound Miner2 [2Fe–2S] clusters quickly became the reduced Miner2 [2Fe–2S] clusters under anaerobic conditions, and further exposure to air led to oxidation of the reduced Miner2 [2Fe–2S] clusters (Fig. 4C). The results suggested that release of NO from the NO-bound Miner2 [2Fe–2S] clusters restores the redox transition activity of the Miner2 [2Fe–2S] clusters.

4. Discussion

In previous studies, we have shown that the mitochondrial matrix protein Miner2 [2Fe–2S] clusters have a unique activity to bind NO and form the stable NO-bound [2Fe–2S] clusters [7]. Here, we report that the NO-bound [2Fe–2S] clusters in Miner2 are light sensitive. Upon the light excitation, the NO-bound Miner2 [2Fe–2S] clusters release NO and become the reduced Miner2 [2Fe–2S] clusters under anaerobic conditions. Additional studies further reveal that binding of NO effectively inhibits the redox transition of the Miner2 [2Fe–2S] clusters, indicating that NO may modulate the physiological function of Miner2 in mitochondria by directly binding to the CDGSH-type [2Fe–2S] clusters.

NO has a high reactivity with heme and iron-sulfur clusters in proteins [32,33]. The binding of NO to heme has been extensively investigated in proteins such as soluble guanylate cyclase [34]. However, much less has been known about the specific interactions between NO and iron-sulfur clusters in proteins. Previous studies from different research groups including ours have shown that iron-sulfur clusters in proteins are readily disrupted by NO, forming the dinitrosyl iron complex [18,21,22], the thiolate-bridged diiron tetranitrosyl complex [23], or the octa-nitrosyl cluster [24]. To the best of our knowledge, the Miner2 CDGSH-type [2Fe–2S] clusters represent the first example that the [2Fe–2S] clusters can bind NO and form stable NO-bound clusters [7]. The unique NO binding activity of the CDGSH-type [2Fe–2S] cluster in Miner2 is likely due to its unusual ligand arrangement of three cysteine and one histidine residues [8]. In the CDGSH-type [2Fe–2S] clusters, one of the iron atoms is ligated via two cysteine residues and the other iron via one cysteine and one histidine residues [8]. The iron center ligated via cysteine/histidine residues in the [2Fe–2S] cluster is most likely redox active [35]. In this context, we

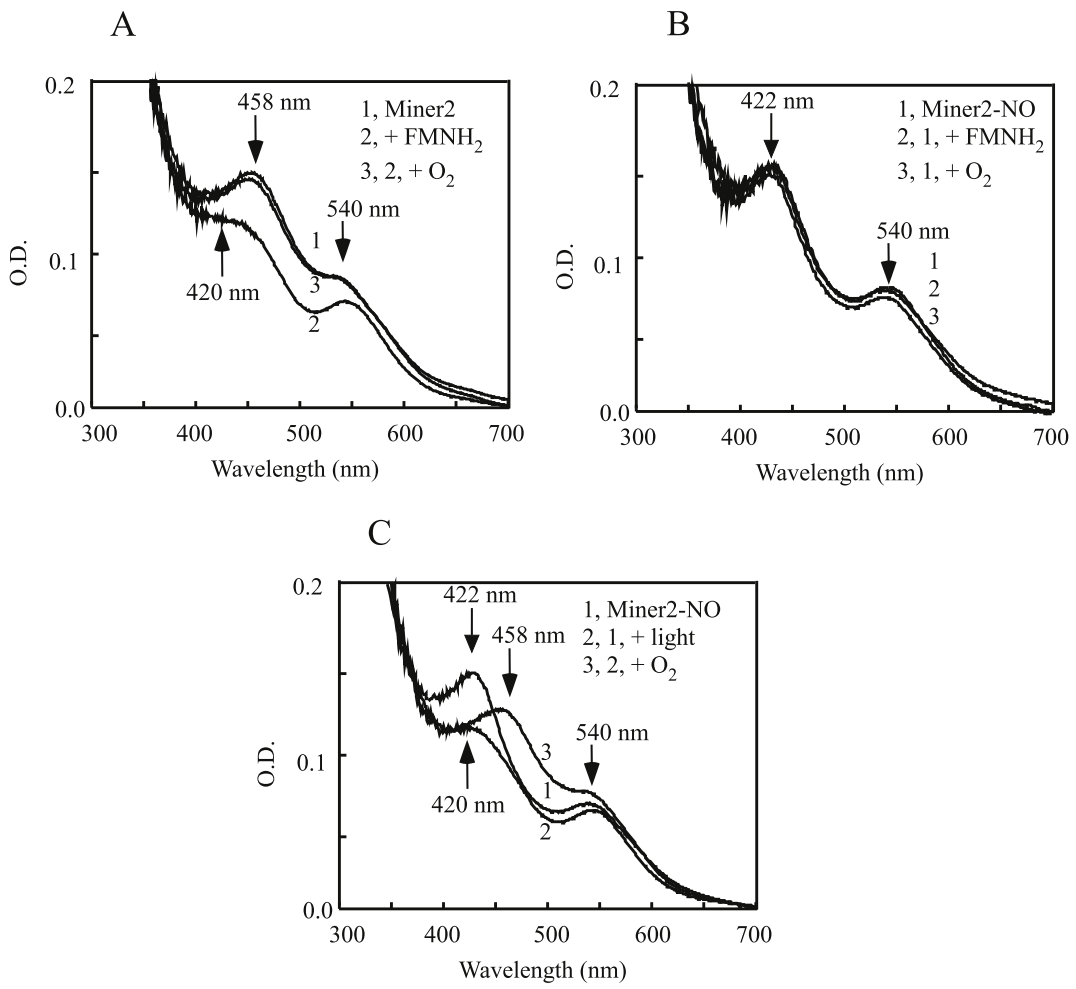

Fig. 4. NO inhibits the redox transition of the Miner2 [2Fe–2S] clusters. A), reduction and oxidation of the Miner2 [2Fe–2S] clusters. Purified Miner2 (10 μM) (spectrum 1) was incubated with FMN (0.1 μM), NADH (20 μM) and *E. coli* flavin reductase (0.1 μM) for 5 min under anaerobic conditions (spectrum 2). The sample was then exposed to air for 10 min (spectrum 3). **B)**, effect of oxygen and $FMNH_2$ on the NO-bound Miner2 [2Fe–2S] clusters. The NO-bound Miner2 [2Fe–2S] clusters (10 μM) (spectrum 1) was incubated with FMN (0.1 μM), NADH (20 μM) and *E. coli* flavin reductase (0.1 μM) for 5 min under anaerobic conditions (spectrum 2). The sample was then exposed to air for 10 min (spectrum 3). **C)**, oxidation of the NO-bound Miner2 [2Fe–2S] clusters by the light excitation and air. The NO-bound Miner2 [2Fe–2S] clusters (10 μM) (spectrum 1) were exposed to the light excitation (400K Lux) for 30 s under anaerobic conditions (spectrum 2), followed by exposure to air for 10 min (spectrum 3). Absorption peaks at 422 nm, 420 nm, and 458 nm represent the NO-bound Miner2 [2Fe–2S] clusters, the reduced Miner2 [2Fe–2S] clusters, and the oxidized Miner2 [2Fe–2S] clusters, respectively.

propose that NO binds to the ferrous iron in the reduced Miner2 [2Fe–2S] cluster (Fig. 5), which is reminiscent of the NO binding in the ferrous heme in proteins [34]. The NO binding affinity for the ferrous heme in proteins is extremely high with the binding constant of about $10^{11}\,M^{-1}$ [36]. On the other hand, the NO binding affinity for the ferric heme in proteins is relatively weaker with the binding constant in the range of 10^{3} to $10^{5}\,M^{-1}$ [37]. Accordingly, we find that the oxidized Miner2 [2Fe–2S] clusters fail to bind NO, and only the reduced Miner2 [2Fe–2S] clusters are able to bind NO with a high binding affinity [7]. Since the redox midpoint potent (E_m) of the CDGSH-type [2Fe–2S] clusters in mitoNEET is about 0 mV at pH 7.0 [38], it is most likely that the [2Fe–2S] clusters in Miner2 are in a reduced state in mitochondria [7]. Thus, the Miner2 [2Fe–2S] clusters are capable of binding NO under physiological conditions.

While the NO-bound Miner2 [2Fe–2S] clusters are stable under aerobic or anaerobic conditions, they are highly sensitive to visible light (Fig. 1). The mass spectrometry analyses showed that two NO molecules may be released from the NO-bound Miner2 [2Fe–2S] clusters by the light excitation (Fig. 2B). The EPR and UV–visible absorption measurements further revealed that the NO-bound Miner2 [2Fe–2S] clusters are converted to the reduced Miner2 [2Fe–2S] clusters upon the light excitation under anaerobic conditions (Fig. 3), demonstrating that the NO binding in the reduced Miner2 [2Fe–2S] clusters is reversible. We were unable to observe the re-binding of NO to the reduced Miner2 [2Fe–2S] clusters after the light excitation, likely due to the diffusion of NO and/or the reaction of NO with other reactants in the solution. However, addition of extra NO to the reaction solution resulted in re-binding of NO to the reduced Miner2 [2Fe–2S] clusters in dark (data not shown), further supporting a notion that the reduced Miner2 [2Fe–2S] clusters can reversibly bind NO.

Like the mitoNEET [2Fe–2S] clusters, the Miner2 [2Fe–2S] clusters can be readily reduced by $FMNH_2$ and oxidized by oxygen. However, the NO-bound Miner2 [2Fe–2S] clusters are resistant to reduction or oxidation (Fig. 4B). When NO is released from the NO-bound Miner2 [2Fe–2S] clusters by the light excitation, the reduced Miner2 [2Fe–2S] clusters can be re-oxidized by oxygen (Fig. 4C), suggesting that NO binding effectively blocks the redox transition of the Miner2 [2Fe–2S] clusters (Fig. 5). It has been reported that the mononuclear iron center in nitrile hydratase from *Rhodococcus* sp. N-771 has a strong binding activity for NO [39]. Purified nitrile hydratase is inactive due to NO binding in the mononuclear iron center, and light excitation releases NO from nitrile hydratase and activates the enzyme activity [40]. For

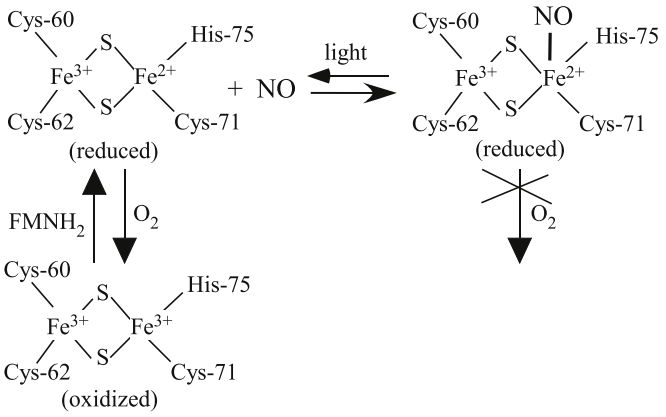

Fig. 5. Proposed model for the NO-mediated inhibition of the redox transition of the Miner2 [2Fe–2S] clusters. Mitochondrial protein Miner2 contains two [2Fe–2S] clusters. Only one of the [2Fe–2S] clusters is shown. In the reduced Miner2 [2Fe–2S] cluster, the iron atom ligated with his/cys is in ferrous state and can be oxidized by oxygen. When NO binds to the ferrous iron atom in the reduced [2Fe–2S] cluster, the cluster can no longer be oxidized by oxygen or reduced by $FMNH_2$. When NO is released from ten NO-bound Miner2 [2Fe–2S] clusters by light excitation, the oxidation of the reduced Miner [2Fe–2S] clusters by oxygen is restored.

the NO-bound Miner2 [2Fe–2S] clusters, release of NO by light excitation appears to also restore the redox transition of the Miner2 [2Fe–2S] clusters. While the mechanism by which the light-induced release of NO from the NO-bound Miner2 [2Fe–2S] clusters is not fully understood, our results strongly suggested that the NO binding in the reduced Miner2 [2Fe–2S] clusters is reversible, and NO may change the physiological activity of Miner2 in mitochondria by directly binding to the [2Fe–2S] clusters. Since there are many CDGSH-type [2Fe–2S] proteins found in human cells [4], the reversible NO binding in the Miner2 [2Fe–2S] clusters may represent a novel example of NO regulation via the CDGSH-type [2Fe–2S] clusters in proteins.

Conflicts of interest

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

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