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Cluster Exchange Reactivity of [2Fe-2S] Cluster-Bridged Complexes of BOLA3 with Monothiol Glutaredoxins

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Abstract

The [2Fe-2S] cluster-bridged complex of BOLA3 with GLRX5 has been implicated in cluster trafficking, but cluster exchange involving this heterocomplex has not been reported. Herein we describe an investigation of the cluster exchange reactivity of holo BOLA3-GLRX complexes using two different monothiol glutaredoxins, *H.s.* GLRX5 and *S.c.* Grx3, which share significant identity. We observe that a 1:1 mixture of apo BOLA3 and glutaredoxin protein is able to accept cluster from donors such as ISCU and a [2Fe-2S](GS)$_4$ complex, with preferential formation of the cluster-bridged heterodimer over the plausible holo homodimeric glutaredoxin. Holo BOLA3-GLRX5 transfers cluster to apo acceptors at rates comparable to other Fe-S cluster trafficking proteins. Isothermal titration calorimetry experiments with apo proteins demonstrated a strong binding of BOLA3 with both GLRX5 and Grx3, while binding with an alternative mitochondrial partner, NFU1, was weak. Cluster exchange and calorimetry experiments resulted in very similar behavior for yeast Grx3 (cytosolic) and human GLRX5 (mitochondrial), indicating conservation across the monothiol glutaredoxin family for interactions with BOLA3 and supporting a functional role for the BOLA3-GLRX5 heterocomplex relative to the previously proposed BOLA3-NFU1 interaction. The results also demonstrate rapid formation of the heterocomplexed holo cluster via delivery from a glutathione-complexed cluster, again indicative of the physiological relevance of the [2Fe-2S](GS)$_4$ complex in the cellular labile iron pool.

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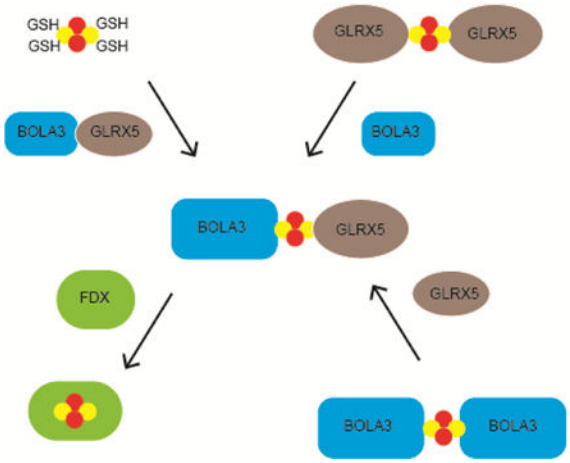

Cluster exchange reactivity of a [2Fe-2S]-bridged BOLA3-GLRX5 heterodimer complex with mitochondrial partner proteins is quantitatively evaluated, and exchange rate constants and ITC-determined thermodynamic parameters contrasted with the corresponding BOLA3 heterocomplex with NFU1. Rapid formation of the holo heterocomplexes via cluster delivery from a glutathione-complexed cluster supports the physiological relevance of the $[2Fe\text{-}2S](GS)_4$ complex in the cellular labile iron pool.

Introduction

The core iron-sulfur cluster assembly pathway (ISC) for iron-sulfur cluster biogenesis involves cluster assembly on an ISCU-type scaffold protein with the involvement of other protein complexes. Subsequently the cluster is transferred to putative acceptors by intermediate cluster carriers, such as glutaredoxin 5 (GLRX5), a process that can sometimes be facilitated by chaperones.[1] Increasing evidence has suggested that BOLA-type proteins are important partners of such monothiol glutaredoxins.[2] Human cells possess three BOLA proteins, namely, mitochondrial BOLA1 and BOLA3, and the cytosolic BOLA2. Missense mutations in BOLA3 have been shown to result in MMDS (Multiple Mitochondrial Dysfunctions Syndromes) in a few patients,[3] with the disease phenotype indicating impairment of certain [4Fe-4S] cluster-containing proteins. The mitochondrial BOLA1 and BOLA3 have been implicated in the maturation of certain [4Fe-4S] proteins in yeast,[4] while the yeast homologue of BOLA2 is involved in Aft-mediated iron regulation.[5]

Evidence for the physiological relevance of such interactions include the co-occurrence of monothiol GLRX and BOLA genes[6] and the detection of a physical interaction between the proteins via high throughput approaches,[2] supporting a functional role for BOLA-GLRX heterodimeric complexes in vivo. In vitro experiments have demonstrated that BOLA2 or BOLA1 homologues can form heterocomplexes involving a bridging [2Fe-2S] cluster with monothiol glutaredoxins from yeast, humans, and *A. thaliana.*[7–9] However, the role of such cluster-bridging heterodimers is not yet clear in humans. Melber et. al have implicated BOLA1 in a preferential interaction with GLRX5, and proposed the interaction of BOLA3 with another Fe-S protein, NFU1, to form a BOLA3-NFU1 heterodimer to facilitate [4Fe-4S] cluster transfer.[10] A later report however found the [2Fe-2S]-bridged BOLA1-

GLRX5 heterodimer to have stronger cluster binding and redox activity, suggesting a likely role in electron transfer processes, while the kinetically labile [2Fe-2S] BOLA3-GLRX5 heterocomplex was concluded to be better suited for Fe-S cluster trafficking.[2]

Most reports of cluster exchange involving [2Fe-2S]-bridged BOLA-GLRX heterocomplexes have been performed with BOLA2. The apo Bol2-Grx3 heterocomplex from *S. cerevisiae* was found to be capable of accepting a [2Fe-2S] cluster from the IscA protein.[11] In vitro experiments found that the bridging [2Fe-2S] cluster of the human GLRX3-BOLA2 heterodimer can be transferred to anamorsin, a protein implicated in the cytosolic iron-sulfur assembly pathway (CIA),[8] and was also supported by in-vivo data.[12] The Bol2-Grx3-bound cluster can also be transferred to Aft2, a protein responsible for iron regulation in yeast.[5] The [2Fe-2S]-containing holo Bol1-Grx4 heterocomplex from *E. coli* was found to transfer cluster to Fdx, but at a slower rate than homo Grx4.[13] However, no such cluster exchange involving a [2Fe-2S]-bridged BOLA3-GLRX heterodimer has yet been reported.

Herein, we report the results of an investigation aimed at understanding the cluster exchange reactivity of cluster-bridged BOLA3-GLRX5 and BOLA3-Grx3 heterodimers (we have used the human BOLA3 and GLRX5 proteins and Grx3 from *S. cerevisiae*), relative to the BOLA3-NFU1 complex. In addition to standard in-vitro chemical reconstitution methods, the [2Fe-2S] cluster-bridged BOLA3-GLRX5 and BOLA3-Grx3 complexes could be formed by cluster delivery from a donor protein, such as ISCU, and by direct delivery from a glutathione-complexed Fe-S cluster, $[2Fe\text{-}2S](GS)_4$. The holo BOLA3-GLRX5 and BOLA3-Grx3 heterodimers were found to transfer cluster to apo proteins, such as FDX1 and FDX2, at rates similar to those reported for proteins involved in cluster trafficking. Isothermal titration calorimetry experiments performed with apo Grx3/GLRX5 and BOLA3 proteins indicated a reasonable binding of BOLA3 with Grx3 and GLRX5, but only very weak binding of BOLA3 with NFU1. Both cluster exchange studies and calorimetry experiments show very similar patterns of behavior for yeast Grx3 (a cytosolic monothiol glutaredoxin) and human GLRX5 (a mitochondrial monothiol glutaredoxin), indicating functional conservation across the monothiol glutaredoxin family of proteins with regard to the interaction with BOLA3. However, quantitative assessment of kinetic and binding parameters for interaction of BOLA3 with NFU1, and the lack of reactivity from the complex, suggest human BOLA3 and NFU1 to be incompatible as partners.

Experimental

Materials

The genes for human BOLA3 in a pET28b(+) vector (UniProt: Q53S33; residues 25–107)[4] and for human glutaredoxin 5 (GLRX5) located in a pET28b(+) vector, both between the NdeI and HindIII restriction sites and lacking the respective mitochondrial targeting sequences (Δ1–24 for BOLA3 and Δ1–31 for GLRX5), were purchased from GenScript.

Protein expression and purification

BL21-DE3 *E. coli* cells with the BOLA3 plasmid were grown in 3 L Luria Bertani (LB) broth media containing kanamycin (30 mg/L) at 37 °C for 7 h. The cultures were then induced with 100 mg/L IPTG and the cell culture incubated overnight at 30 °C. Cells were harvested by centrifugation at 5000 rpm for 15 min, suspended in 30 mL of 50 mM HEPES, 100 mM NaCl, pH 7.5, and lysed by use of a dismembranator. The lysate was centrifuged at 15000 rpm for 30 min, the supernatant passed over a Ni-NTA column, and the protein eluted with buffer containing 0.5 M imidazole, after 3 washes with the HEPES buffer. The isolated protein was concentrated by amicon ultrafiltration and subsequently desalted by passing through a PD-10 column. Total protein concentrations were determined by use of the Bradford assay. *H.s.* GLRX5, lacking the first 31 amino acids (Δ1–31) that correspond to the mitochondrial targeting sequence,[14] was expressed and purified as reported previously.[15] *S.c.* Grx3 (Q03835, residues 36–285), in an open reading frame containing both the Trx-like and Grx-like domains, but lacking the first 35 amino acids (Δ1–35[7]) in a pET28b(+) vector, was expressed and purified from *E. coli* BL21 (DE3).[16]

Human ISCU (*H.s.* ISCU) and *Thermotoga maritima* (*T.m.* NifS) were purified as previously reported.[17–19] The plasmid for human ferredoxin-1 (*H.s.* FDX1) was kindly provided by J. Markley, and the protein was expressed and purified by the procedures described before.[20] Human ferredoxin-2 (*H.s.* FDX2) was expressed and purified as previously reported.[21] The ferredoxins purified as holo proteins and were then subsequently converted to apo forms by treatment with 100 mM EDTA, 5 mM DTT, and 8 M urea in 50 mM HEPES, 100 mM NaCl, pH 7.5 and heating to 40 °C. The colorless solution was subsequently passed through a PD-10 column to remove the excess reagents. Expression and purification of human NFU1 were performed from BL21 (DE3) competent cells as previously described.[22, 23] Concentrations of all the apo proteins were estimated by the use of the Bradford Assay.

In vitro reconstitution of apo proteins

All in vitro reconstitutions were performed in 50 mM HEPES, 100 mM NaCl, pH 7.5. Reconstitutions of the BOLA3-GLRX5 and BOLA3-Grx3 heterodimers were conducted as previously described,[2] using a 1:1 molar ratio (350 μM of each protein) in the presence of 2 μM *T.m.* NifS, 5 mM DTT, and 3 mM GSH. The reaction mixture was argon-purged for 30 min, and then made up to 0.6 mM in Fe^{3+} and 0.6 mM in L-cysteine. Following incubation for 1.5 h, the reaction mixture was passed through a PD-10 desalting column to remove excess reactants. The total concentration of protein was estimated by the Bradford assay, and the effective holo concentration of [2Fe-2S] BOLA3-GLRX(Grx) protein was estimated by use of the extinction coefficient, ε_{393}= 8.3 mM^{-1} cm^{-1}, as reported in literature.[2, 7] Holo GLRX5 and Grx3 were also reconstituted by themselves (without BOLA3), by a similar method.[15, 16]

Reconstitution of full-length human NFU1 was performed as previously described,[24] where the reconstitution mixture contained 200 μM protein, 5 mM DTT, 2 μM *T.m.* NifS, 1 mM Fe^{3+}, and 3 mM cysteine. After incubating for 45 min, the holo protein was isolated following passage through a PD-10 column. Reconstitution of BOLA3 alone, to form holo

homodimeric BOLA3, was performed using the same method as for NFU1. For ISCU, 200 μM of apo protein was incubated with 50 mM DTT and 8 M urea, argon purged for 30 min, and then made up to 1 mM in Fe^{3+} and 1 mM in S^{2-}. After incubation for 1 h, the reaction mixture was concentrated and passed through a PD-10 desalting column, equilibrated with 50 mM HEPES, 100 mM NaCl, pH 7.5, to remove excess reagents.

Circular dichroism spectra of reconstituted BOLA3-GLRX5 and BOLA3-Grx3

Jasco J-815 circular dichroism spectrometer was used to measure the circular dichroism spectra of the reconstituted holo BOLA3-GLRX5 and BOLA3-Grx3 heterodimeric proteins. Spectra were obtained from 300 - 600 nm at a 200 nm/min scan rate and were subsequently deconvoluted by use of the deconvolution function from the Spectramanager II analysis software. The software uses overlapping Lorentzian curves having the same full width at the half-maximum value to accurately distinguish the peak positions for each band, using Fourier Self Deconvolution (FSD) and Finite Impulse Response Operator (FIRO) methods.[25, 26]

$[2Fe\text{-}2S](GS)_4$ cluster uptake to form [2Fe-2S]-bridged BOLA3-GLRX5 and BOLA3-Grx3 heterodimers

The $[2Fe\text{-}2S](GS)_4$ cluster used was synthesized as previously reported.[27] For cluster uptake, a 100 μM solution of $[2Fe\text{-}2S](GS)_4$ in 50 mM HEPES, 100 mM NaCl, pH 7.5 buffer was added to an equimolar mixture of 50 μM apo GLRX5 or Grx3 protein and 50 μM apo BOLA3 in the presence of 0.2 mM DTT. The reaction was followed by monitoring the appearance of a distinct negative peak at 350 nm in the CD spectra (a characteristic of the cluster-bound heterodimer) obtained under anaerobic conditions, and the signal intensity from 345–355 nm was measured for a period of 30 min, with 10 s between each scan. Data were converted to the percentage of cluster transfer and analyzed by use of the DynaFit program[28] to determine the second-order rate constants for the reaction by best-fit simulation to second-order kinetics. The signal for the holo protein at the end of the reaction was compared to the reconstituted holo protein signal for the same concentration of [2Fe-2S] cluster concentration to determine the percentage of cluster transfer.

$[2Fe\text{-}2S](GS)_4$ cluster uptake by an equimolar mixture of BOLA3 and NFU1

The aforementioned procedure for formation of the BOLA3-GLRX5 heterodimer was similarly followed with an equimolar mixture of NFU1 and BOLA3.

Cluster transfer experiments analyzed by circular dichroism spectroscopy

UV-vis circular dichroism spectroscopy was used to investigate cluster transfer from holo iron-sulfur cluster proteins to apo proteins using 1-cm quartz cuvettes. CD scans from 600 - 300 nm were collected under anaerobic conditions, at a scan rate of 200 nm/min with a 2 min interval between accumulations. A mixture of apo protein in 5 mM DTT and 3 mM GSH in 50 mM HEPES, 100 mM NaCl, pH 7.5 was degassed, transferred to an anaerobic cuvette via a gas tight syringe, and the reaction initiated via the addition of the degassed holo protein. The concentrations of [2Fe-2S] cluster for each holo protein were determined via absorbance measurements. Data were processed by use of JASCO Spectramanager II

Analysis software, and the deconvolution function from Spectramanager II analysis software was used for analysis of the bands in the spectra that contained overlapping Lorentzian curves with the same full width at half-maximum value to accurately distinguish the peak positions for each band. The second-order rate constants were calculated using the program DynaFit by BioKin[28], by a method similar to that developed by Johnson and colleagues.[11, 29] Proteins were mixed in a 1:1 ratio (30 μM each) and the characteristic peaks for the respective holo acceptor protein (445 nm for FDX1, 440 nm for FDX2, 350 nm for BOLA3-GLRX5, 355 nm for BOLA3-Grx3 and 363 nm for NFU1) were monitored by CD. The data were converted to percent cluster transfer, where the percentage of cluster transferred was determined by comparing the signal intensity for the holo protein after cluster transfer to that of control spectra of the holo protein with the same cluster concentration following reconstitution.

Isothermal titration calorimetry for interaction between apo BOLA3 and apo GLRX5/Grx3 or NFU1

Apo BOLA3 and GLRX5 or Grx3 proteins were dialyzed overnight in 50 mM HEPES, 100 mM NaCl, 5 mM GSH, pH 7.5 at 4 °C. GSH was added as it has been found to mediate the interaction between BOLA3 and GLRX5.[4] Apo BOLA3 and apo NFU1 were also dialyzed in 50 mM HEPES, 100 mM NaCl, pH 7.5. ITC experiments were carried out at 30 °C using a MicroCal calorimeter. The sample cell contained 50 μM apo Grx3, GLRX5, or NFU1 in the abovementioned buffer used for dialysis, and the syringe contained 1 mM BOLA3 in the same buffer. 1 mM BOLA3 was titrated in 10 μL aliquots with 24 s durations and intervals of 6 min. Control titrations of buffer into protein and protein into buffer were also performed and the data subtracted from the raw experimental output. The data was analyzed using the Origin Software provided by MicroCal.

Results

BOLA3 has previously been reported to form a functional heterodimer with the Fe-S cluster protein NFU1.[10] However, GLRX5 has also been proposed to form a [2Fe-2S]-bridged heterodimeric complex with BOLA3 that is able to promote [2Fe-2S] cluster trafficking.[2] To begin to address the scope of binding partners and their functional relevance, we have performed detailed investigations with human BOLA3 to determine the preferred binding partners and possible pathways for Fe-S cluster transfer. Both *H. sapiens* GLRX5 and *S. cerevisiae* Grx3 were used to investigate BOLA3-GLRX(Grx) interactions and the cluster transfer reactivity of the [2Fe-2S] cluster-bound BOLA-Grx heterodimer. Though cytosolic Grx3 is unlikely to interact with the mitochondrial BOLA3 in vivo, we chose to explore the influence of functional conservation across the monothiol glutaredoxin family with regard to an interaction with BOLA3. *S. cerevisiae* Grx3 displays 49% sequence identity with human GLRX5, while having an extra thioredoxin domain that is independent of its cluster binding glutaredoxin domain. The human homologue Grx3 has an additional glutaredoxin domain, although only one cluster binding glutaredoxin domain is sufficient for in vitro cluster transfer,[30] and thus, *S.c.* Grx3 was chosen as a simpler model.

Reconstitution and formation of BOLA3-Glutaredoxin heterocomplex

Reconstitution of an equimolar mixture of GLRX5/Grx3 and BOLA3 resulted in the formation of a [2Fe-2S]-bridged BOLA3-glutaredoxin heterodimer complex that exhibited characteristic UV and CD signatures (Figure 1), consistent with similar complexes reported in the literature, with a pronounced negative feature at 350 nm.[2, 7] Holo GLRX5/Grx3 also has a negative feature at 350 nm, but it is not as pronounced as that observed with the heterocomplex. [4, 7, 31] Accordingly, this negative feature distinguishes the holo heterocomplex from the holo homodimeric GLRX5/Grx3.

We were successful in reconstituting BOLA3 to form a holo homodimer (without a partner Grx) to form holo BOLA3; however, the reconstituted BOLA3 lacks any prominent CD response (data not shown), most likely reflecting the absence of substantial chirality around the bound cluster as previously observed with Grx proteins.[15, 16] This form of holo homodimeric BOLA 3 is under further investigation. The BOLA3-GLRX5/Grx3 heterocomplex formed very rapidly, within the first 2 min, after which no additional significant spectral changes were observed (Figure 2) when either apo BOLA3, or apo GLRX5/Grx3, was added to the other partner (holo GLRX5/Grx3 or holo BOLA3, respectively). This indicates that the cluster-bridged heterodimer is thermodynamically more stable than the corresponding cluster-bridged homodimer, and confirm the results obtained from studies of the yeast BOLA2-Grx3 heterodimer.[7]

Cluster transfer from holo heterocomplex to apo ferredoxins

Reconstituted BOLA3-GLRX5 and BOLA3-Grx3 heterocomplexes were found to transfer cluster to both apo FDX1 and FDX2 (Figures 3,4) with rate constants (Table 1) comparable to other donor transfers to FDX proteins,[15, 32] including homodimeric Grx3 and GLRX5 (e.g., 2100 ± 500 M^{-1} min^{-1} for cluster transfer from homodimeric Grx3 to FDX1 and 2800 ± 380 M^{-1} min^{-1} for heterodimeric BOLA3-Grx3 to FDX1) .
15, 32 15, 32 15, 32 15, 32 15, 32 15, 32

Cluster uptake from holo ISCU

A 1:1 mixture of apo Grx3/GLRX5 and apo BOLA3 was found to take up [2Fe-2S] cluster from donors, such as holo ISCU, to form the [2Fe-2S]-bridged Grx3/GLRX5-BOLA3 heterocomplex, as monitored by the appearance of the characteristic CD signal of the holo heterocomplex (Figure 5). The rate constants for cluster uptake, 14000 M^{-1} cm^{-1} for BOLA3-GLRX5 and 4600 M^{-1} cm^{-1} for BOLA3-Grx3, are comparable to those observed for other proteins involved in cluster transfer.[32, 33] We have previously shown that apo Grx3 and GLRX5 can also take up cluster from ISCU to form holo homodimeric Grx3 and GLRX5.[15, 16] However, when an equimolar concentration of BOLA3 is present, along with the glutaredoxin, the cluster bound BOLA3-GLRX5 (or BOLA3-Grx3) heterodimer is formed instead of a GLRX5 (or Grx3) homodimer, as seen by the appearance of a CD signature that is different for holo homodimeric Grx3 or GLRX5 (Figures 2A and 2B) as reflected by the presence of a more pronounced negative feature at 350 nm. The rate constants for cluster transfer from holo ISCU to apo BOLA3-GLRX5 and BOLA3-Grx3 (Table 1) are comparable to those determined for transfer from holo ISCU to apo GLRX5 and Grx3;[32, 34] therefore, the alternate possibility for cluster donation from ISCU to apo

Grx/GLRX, leading to subsequent heterodimer formation with BOLA3 can be ruled out. In addition, the reasonably strong binding between apo BOLA3 and Grx3/GLRX5 (see ITC results below) indicates that an equimolar mixture of apo Grx3/GLRX5 and BOLA3 would be predominantly present as a heterodimer, also confirming the proposed model for the GLRX5-BOLA3 interaction.[4] Accordingly, direct cluster donation to an apo BOLA3-Grx3 or BOLA3-GLRX5 heterodimer is more likely.

Cluster exchange involving NFU1

Unlike the formation of the holo BOLA3-Grx heterodimeric species following addition of holo BOLA3 to apo GLRX5/Grx3, or holo GLRX5/Grx3 to apo BOLA3, we observe a fast and quantitative cluster transfer from holo BOLA3 to apo NFU1, as noted by the appearance of the distinct CD signature of holo NFU1 (Figure 6A), but no formation of a BOLA3-NFU1 heterodimeric complex. The reverse reaction, from holo NFU1 to apo BOLA3 does not give rise to any transfer or the formation of any intermediate species, with the CD signature representing only holo NFU1 after 1 h (Figure 6B), indicating that cluster transfer did not occur.

Cluster exchange between BOLA3-GLRX heterodimer and NFU1 were unsuccessful. No cluster transfer was observed from holo BOLA3-GLRX5 (or BOLA3-Grx3) to apo NFU1, as well from holo NFU1 to apo BOLA3-GLRX5 (or BOLA3-Grx3) (data not shown).

Cluster exchange with [2Fe-2S](GS)$_4$

An equimolar mixture of apo BOLA3 and GLRX5/Grx3 was found to rapidly take up cluster from [2Fe-2S](GS)$_4$, as observed by circular dichroism spectroscopy (Figure 7). The [2Fe-2S](GS)$_4$ complex is CD silent, while the [2Fe-2S]-bridged Grx-BOLA3 heterodimer has a distinct CD signature, so cluster uptake was followed by monitoring the enhancement of the negative peak at 350–360 nm over time. The observed rate constants (Table 1) are among the fastest observed for any exchange reaction involving either the glutathione-complexed cluster or the exchange of clusters between cognate proteins. A similar experiment, combining [2Fe-2S](GS)$_4$ with an equimolar mixture of NFU1 and BOLA3, resulted in the formation of holo NFU1, indicative of an inability to form a similar cluster-bound NFU1-BOLA3 complex (Figure 8).

Isothermal titration calorimetry to study apo-apo interactions

Data from ITC experiments for Grx-BOLA3 binding were readily fit to a one-site model, indicating that in the presence of 5 mM GSH, apo GLRX5 binds to BOLA3 with K_D = 8.3 μM ($K_A = 1.2 \times 10^5$ M^{-1}) and N = 1.02. The affinity of apo Grx3 for BOLA3 is similar with K_D = 6.7 μM ($K_A = 1.5 \times 10^5$ M^{-1}) and N = 0.94, (Figure 9), also indicating a 1:1 binding stoichiometry. The K_D values reported here from ITC are in close agreement to that reported previously by microscale thermophoresis (3 μM).[4] However, titration of BOLA3 to NFU1 did not support tight binding, yielding small heat changes that did not saturate and could not be fit to valid parameters (data not shown).

Discussion

In this report, we demonstrate that a [2Fe-2S]-bridged heterodimer formed from the combination of monothiol glutaredoxins GLRX5/Grx3 and BOLA3 is capable of cluster exchange with a variety of proteins. Our experiments indicate that this cluster-bridged heterodimer is preferred over the corresponding holo BOLA3 or holo Grx homodimers, as reconstituted holo forms of BOLA3 and GLRX5/Grx3 are immediately converted into the heterodimer in the presence of the other interacting partner, Cluster uptake experiments from the cluster donor scaffold protein ISCU also support this, indicating that the heterodimeric complex is the more stable form, confirming previous studies performed with BOLA and Grx proteins.[2, 9]

Prior NMR studies have also demonstrated that the formation of the [2Fe-2S] BOLA3-GLRX5 heterocomplex is more favorable than the [2Fe-2S] GLRX5 homodimer.[2] In addition, it has been reported that [2Fe-2S] BOLA3-GLRX5 is intrinsically less stable than the similar [2Fe-2S] BOLA1-GLRX5 complex (BOLA1 and BOLA3 both being mitochondrial proteins). However, molecular modeling has indicated that the cluster in [2Fe-2S] BOLA3-GLRX5 was more accessible and kinetically labile; while the [2Fe-2S]-bridged BOLA1-GLRX5 heterodimer had stronger cluster binding and redox activity. Cluster-bound BOLA1-GLRX5 was therefore suggested to play a role in electron transfer processes, while the kinetically labile [2Fe-2S] BOLA3-GLRX5 heterocomplex was concluded to be more suited for Fe-S cluster trafficking. Indeed, we observe cluster transfer from [2Fe-2S] BOLA3-GLRX5 and [2Fe-2S] BOLA3-Grx3 to apo FDX1 and FDX2, and an uptake of cluster from ISCU and $[2Fe\text{-}2S](GS)_4$ to form [2Fe-2S] BOLA3-Grx complex. There is selectivity in the cluster transfer behavior as transfer from holo NFU1 to either apo BOLA3-GLRX5 or BOLA3-Grx3, as well as from holo BOLA3-GLRX5 or BOLA3-Grx3, to apo NFU1 were unsuccessful. The exact in vivo function of such a BOLA3-GLRX5 heterocomplex is not yet known. GLRX5 is a core component of the Isc pathway of Fe-S cluster biogenesis, but BOLA3-GLRX5 complex formation is not critical for this role.[4] Notably, GLRX5 is at least 4-fold more abundant than BOLA3[2] in yeast mitochondria, so it is likely that GLRX5 should still be predominantly present in a homodimeric form. A possible function of BOLA could be the conversion of a labile [2Fe-2S]-bridged holo GLRX homodimer to a more thermodynamically stable [2Fe-2S]-bridged holo Grx-BOLA heterodimer, as previously suggested.[2, 9]

Based on studies in yeast and the results of in vitro experiments,[4, 10] NFU1 has been implicated in an interaction with BOLA3, where the NFU1-BOLA3 heterodimer has been proposed to be important for the maturation of certain [4Fe-4S] clusters. Interestingly, we observe a much weaker interaction between NFU1 and BOLA3 than between GLRX5/Grx3 and BOLA3 under our experimental conditions. Previous experiments with human apo proteins have shown binding of both BOLA1 and BOLA3 to GLRX5 with a K_D of 3 μM (comparable to our value of 8.3 μM), but a preferential interaction of BOLA1 with holo GLRX5 was suggested. Primarily this was because of the greater stability of the BOLA1-GLRX5 cluster heterodimer toward reduction by dithionite and from the observation that size exclusion chromatography was not able to detect the GLRX5-BOLA3 heterodimer. It was suggested that instead of GLRX5, BOLA3 interacts preferentially with NFU1, as apo

BOLA3 was found to bind to holo NFU1 with ~ 4-fold higher affinity in comparison to Grx5, when binding was investigated by microscale thermophoresis.[4]

The CD signature for the reconstituted BOLA3-GLRX5 heterodimer in the same report demonstrated that GSH is crucial to promote complex formation between BOLA3 and GLRX5, which is also consistent with our observations (data not shown). Binding of apo GLRX5 to apo NFU1 or apo BOLA3 was found to be comparable by microscale thermophoresis; however, the binding of holo NFU1 to apo BOLA3 was found to be stronger. In our hands, we observe negligible binding between NFU1 and BOLA3 in their apo forms, while binding between BOLA3 and GLRX5 was much stronger when monitored by isothermal titration calorimetry. While it was not possible to test the interaction between holo NFU1 and apo BOLA3, under anaerobic conditions, our CD experiments nevertheless show quantitative cluster transfer from holo homodimeric BOLA3 to apo NFU1, in contrast to the formation of a heterocomplex observed with GLRX5/Grx3. The reverse transfer from holo NFU1 to apo BOLA3 and GLRX5/Grx3 was also attempted; however, the CD signature does not indicate formation of any intermediate species in contrast to that formed with GLRX5/Grx3. Although human NFU1 has previously been reported as a [4Fe-4S] protein based on a weak Mossbauer signal,[35] prior reports generally suggest that NFU1 homologues can bind both [2Fe-2S] and [4Fe-4S] clusters.[36–38] while our own studies clearly and rigorously support a bound [2Fe-2S] cluster that can be quantitatively transferred to [2Fe-2S] acceptor proteins.[32, 39] We also demonstrated formation of a [2Fe-2S] cluster-bound NFU1 under NifS mediated reconstitution conditions, with the holo form having a pronounced CD signature. Additionally NFU1 has been demonstrated to be capable of accepting a [2Fe-2S] cluster from several [2Fe-2S] donors.[39] The holo NFU1 CD signature observed in this study resembles that formed upon cluster uptake from [2Fe-2S] donors [39], indicating that there is a transfer of [2Fe-2S] cluster from holo BOLA3 to apo NFU1.

Significantly, however, an effective pathway for the formation of holo heterodimeric BOLA3-glutaredoxin was demonstrated via the glutathione-complexed [2Fe-2S] cluster that we have previously proposed to be a key component of the cellular iron pool, following formation by glutathione extraction from assembly proteins, such as ISCU [27, 40]. The broad range of rapid and physiologically relevant cluster exchange chemistry of this species,[40] combined with the fact that such reactivity is superior, in specific cases, to other natural cluster donor/trafficking proteins,[32] is further demonstrated by the results reported herein, and is again strongly indicative of the physiological relevance of this glutathione-complexed cluster in the chemistry of cellular iron-sulfur cofactors.

In conclusion, our results from isothermal titration calorimetry, circular dichroism spectroscopy, and analysis of the kinetic rate constants indicate that the BOLA3-GLRX5 interaction is significant, while the BOLA3-NFU1 interaction is substantially weaker or negligible. Therefore, the BOLA3-GLRX5 heterocomplex seems to be a more viable physiological candidate for Fe-S cluster trafficking. Interestingly, the behavior of *H.s.* GLRX5 and *S.c.* Grx3 was similar, suggesting a conserved nature of heterodimeric complex formation between BOLA proteins and monothiol glutaredoxins. This is not surprising as there is substantial functional complementarity across the monothiol glutaredoxin family of proteins, with extensive conservation around the cluster-binding site.[41] NMR studies with

human proteins have indicated that the interaction regions of GLRX5, affected upon formation of the [2Fe-2S]-bridged GLRX5-BOLA3 complex, are essentially the same as in the [2Fe-2S]-bridged homodimeric GLRX5.[2, 4] Thus, GLRX5 is expected to behave similarly to Grx3, because the two proteins have significant sequence similarity near the cluster-binding site. Grx3 and BOLA3 proteins are not found in the same cellular compartment; thus, their protein-protein interaction and complex formation indicates the conservation of Fe-S cluster proteins and their interaction network, in addition to the functional importance of the monothiol GLRX-BOLA heterodimers for cellular cluster trafficking.

Acknowledgments

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Significance to Metallomics

Herein, we report that a [2Fe-2S]-bridged BOLA3-GLRX5 is well suited to act as a physiological candidate for Fe-S cluster trafficking, The behaviors of *H.s.* GLRX5 and related *S.c.* Grx3 were similar, suggesting conservation in heterodimeric complex formation between BOL proteins and monothiol glutaredoxins. Our observation of rapid and efficient cluster delivery from a [2Fe-2S](GS)$_4$ complex to form a [2Fe-2S]-bridged BOLA-GLRX5 underlines the physiological relevance of the glutathione complex.

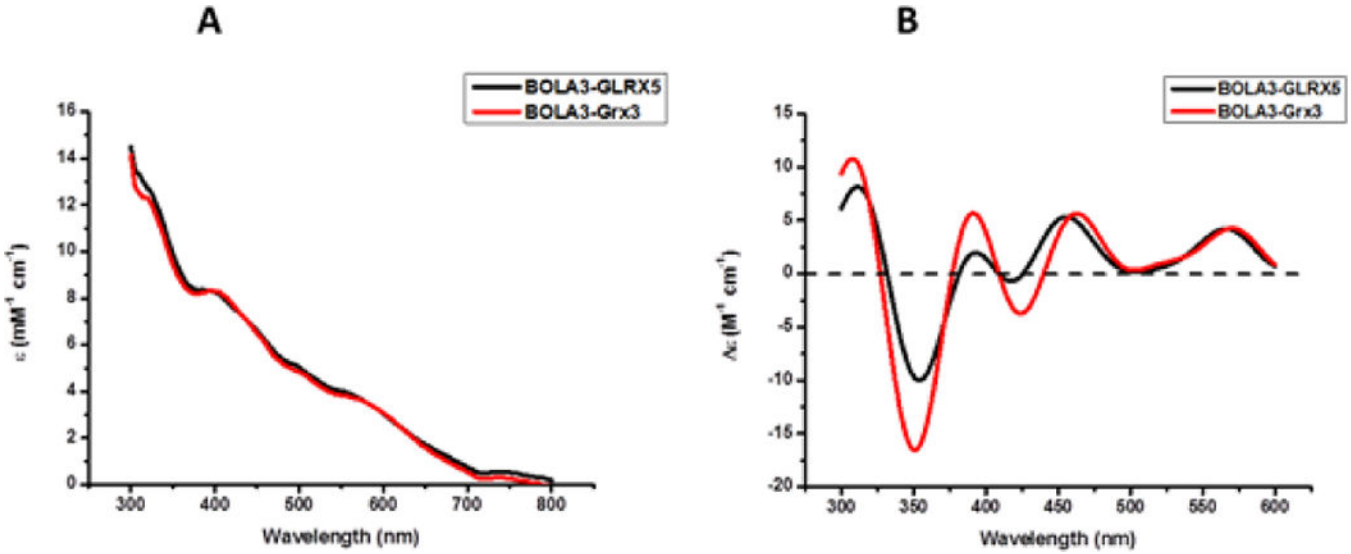

Figure 1.

Comparison of **(A)** UV spectra of holo GLRX5-BOLA3 and Grx3-BOLA3 heterocomplexes obtained by NifS-mediated chemical reconstitution, and **(B)** CD spectra of reconstituted holo GLRX5-BOLA3 and Grx3-BOLA3. The extinction coefficients are based on the cluster concentrations.

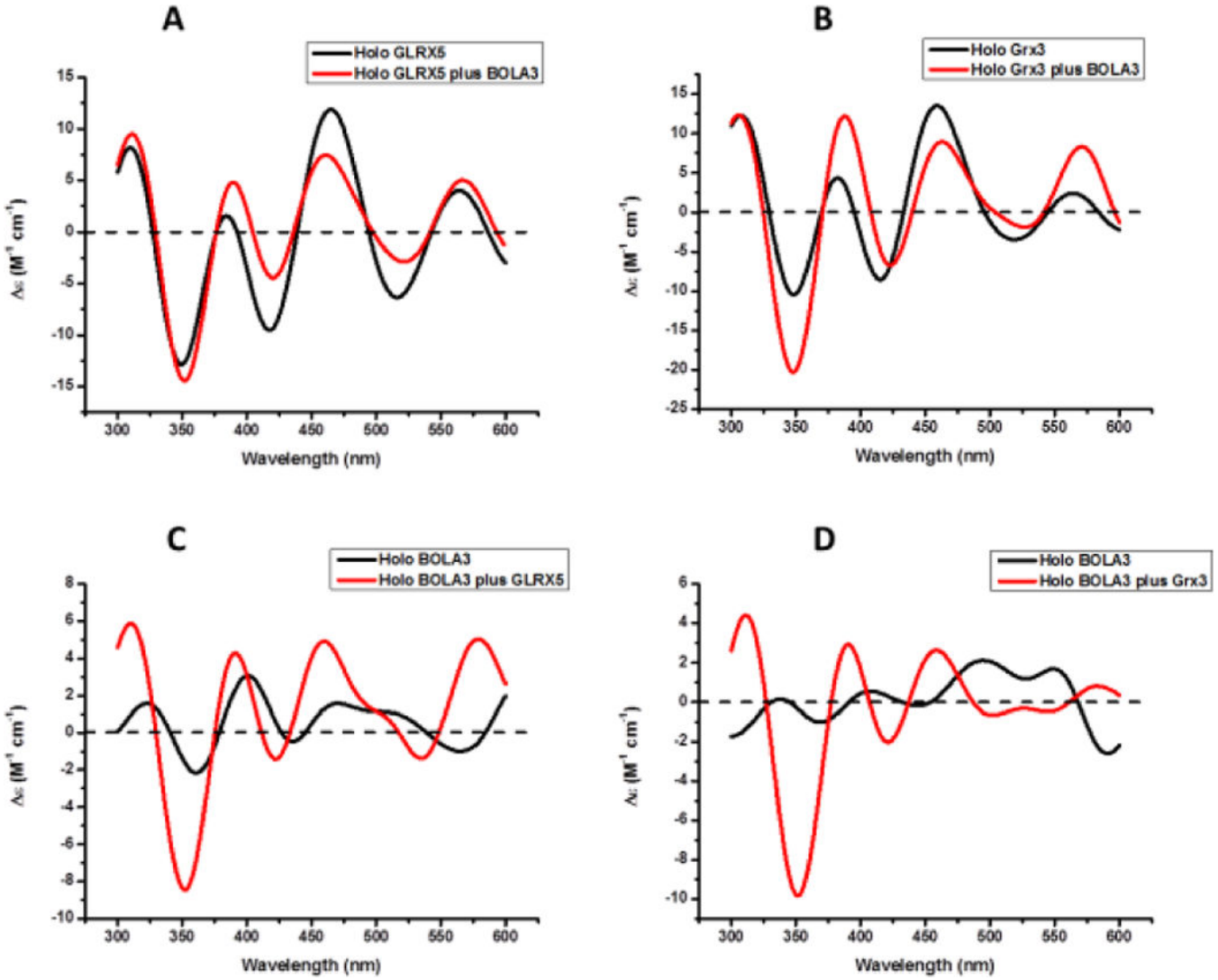

Figure 2.

(A) Formation of BOLA3-GLRX5 holo heterodimer following addition of apo BOLA3 to holo homodimers of GLRX5. **(B)** Formation of BOLA3-Grx3 holo heterodimer complexes following addition of apo BOLA3 to holo homodimers of Grx3. **(C)** Formation of BOLA3-GLRX5 holo heterodimer after addition of apo GLRX5 to holo BOLA3 **(D)** Formation of BOLA3-Grx3 holo heterodimer upon addition of apo GLRX5 or apo Grx3 to holo BOLA3. Holo BOLA3 lacks a well defined CD signature, but immediately changes following addition of either GLRX5 or Grx3.

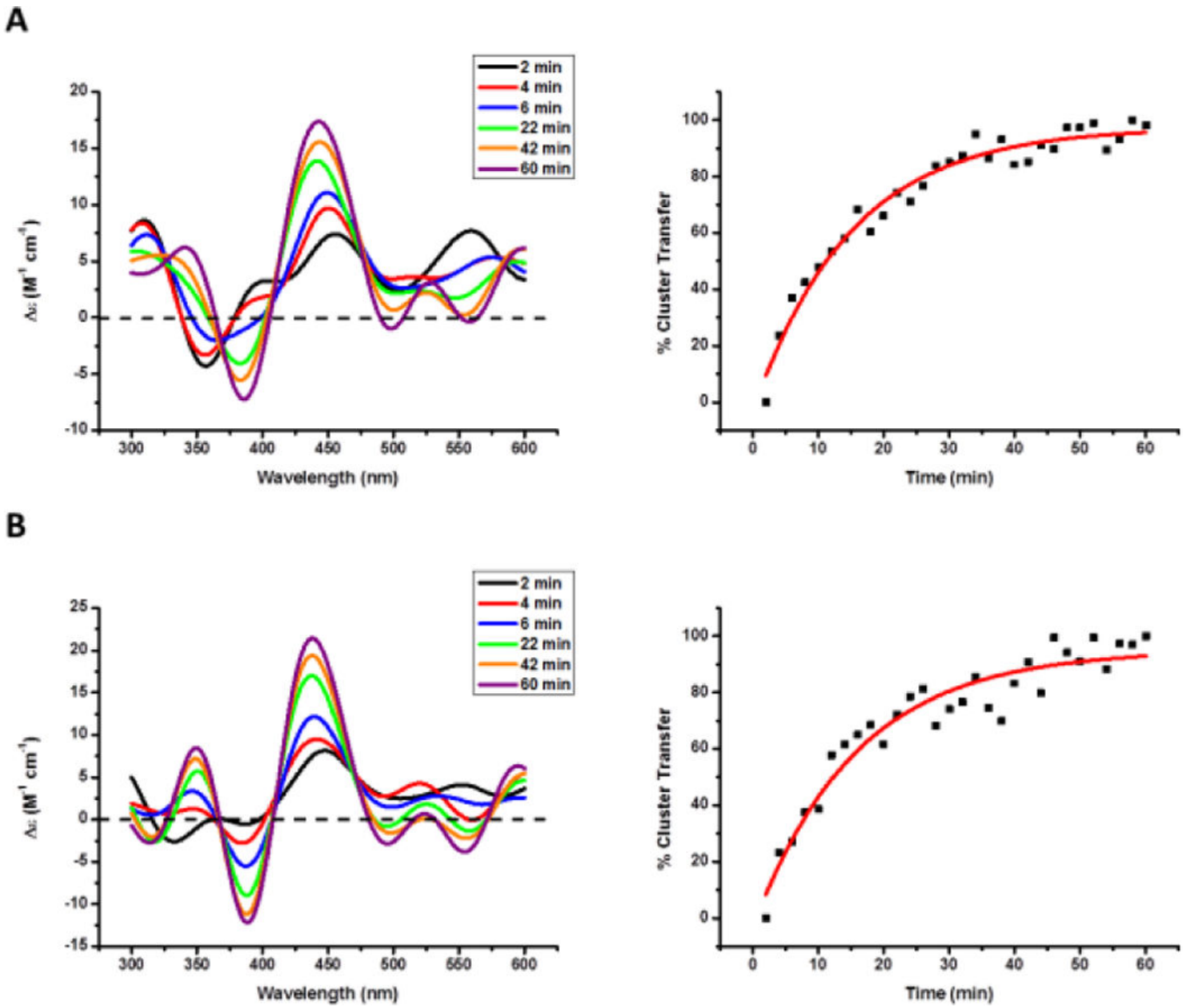

Figure 3.

Time course measurements for cluster transfer from **(A)** holo BOLA3-GLRX5 and **(B)** holo BOLA3-Grx3 to apo FDX2 under anaerobic conditions. The CD signal intensity was monitored at room temperature for a 1:1 donor:acceptor cluster stoichiometry in the presence of 3 mM GSH. CD spectra in the 300–600 nm region were recorded every 2 min.

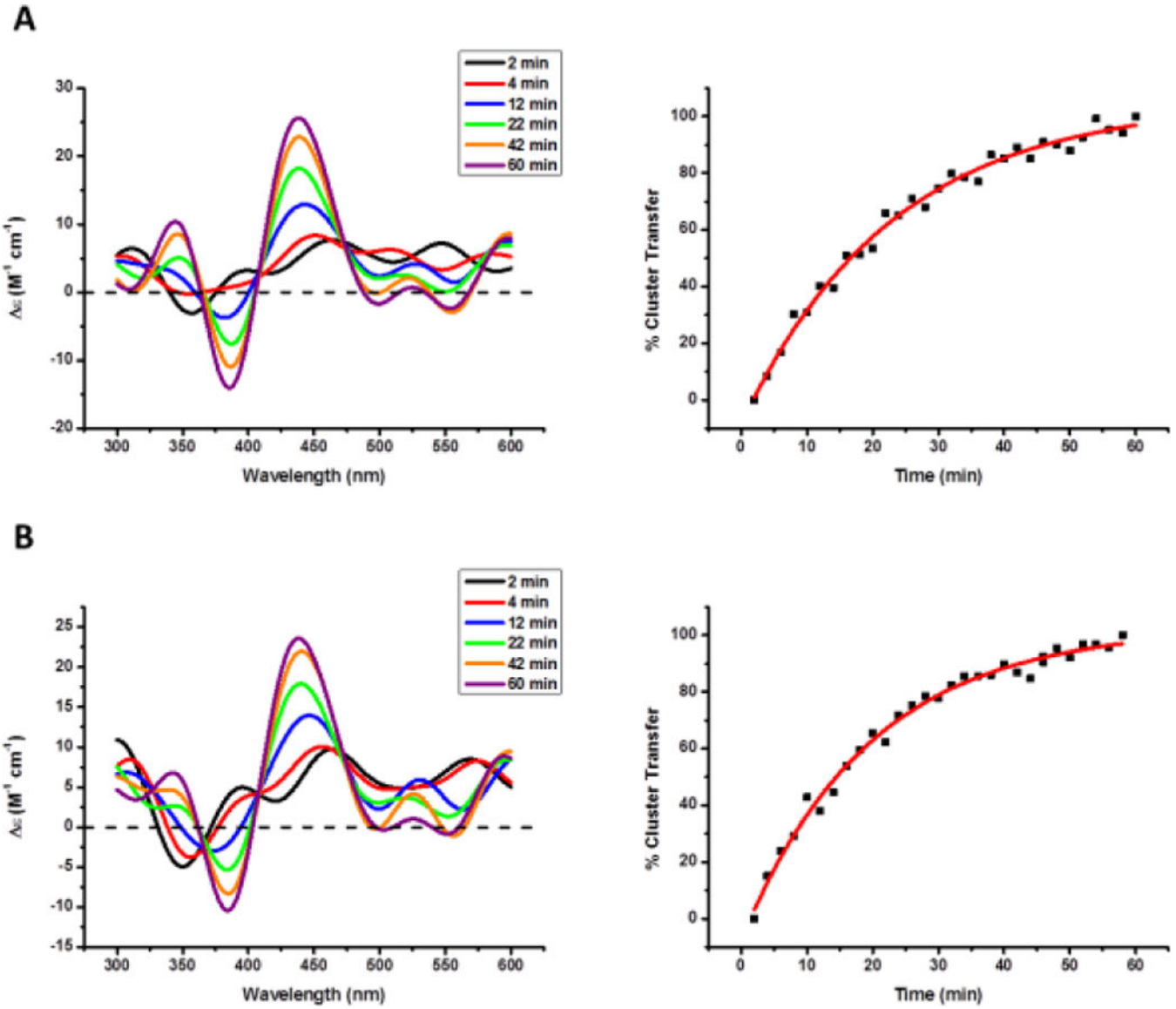

Figure 4.

Time course measurements for cluster transfer from **(A)** holo BOLA3-GLRX5 and **(B)** holo BOLA3-Grx3 to apo FDX1 under anaerobic conditions. The CD signal intensity was monitored at room temperature for a 1:1 donor:acceptor cluster stoichiometry in the presence of 3 mM GSH. CD spectra in the 300–600 nm region were recorded every 2 min.

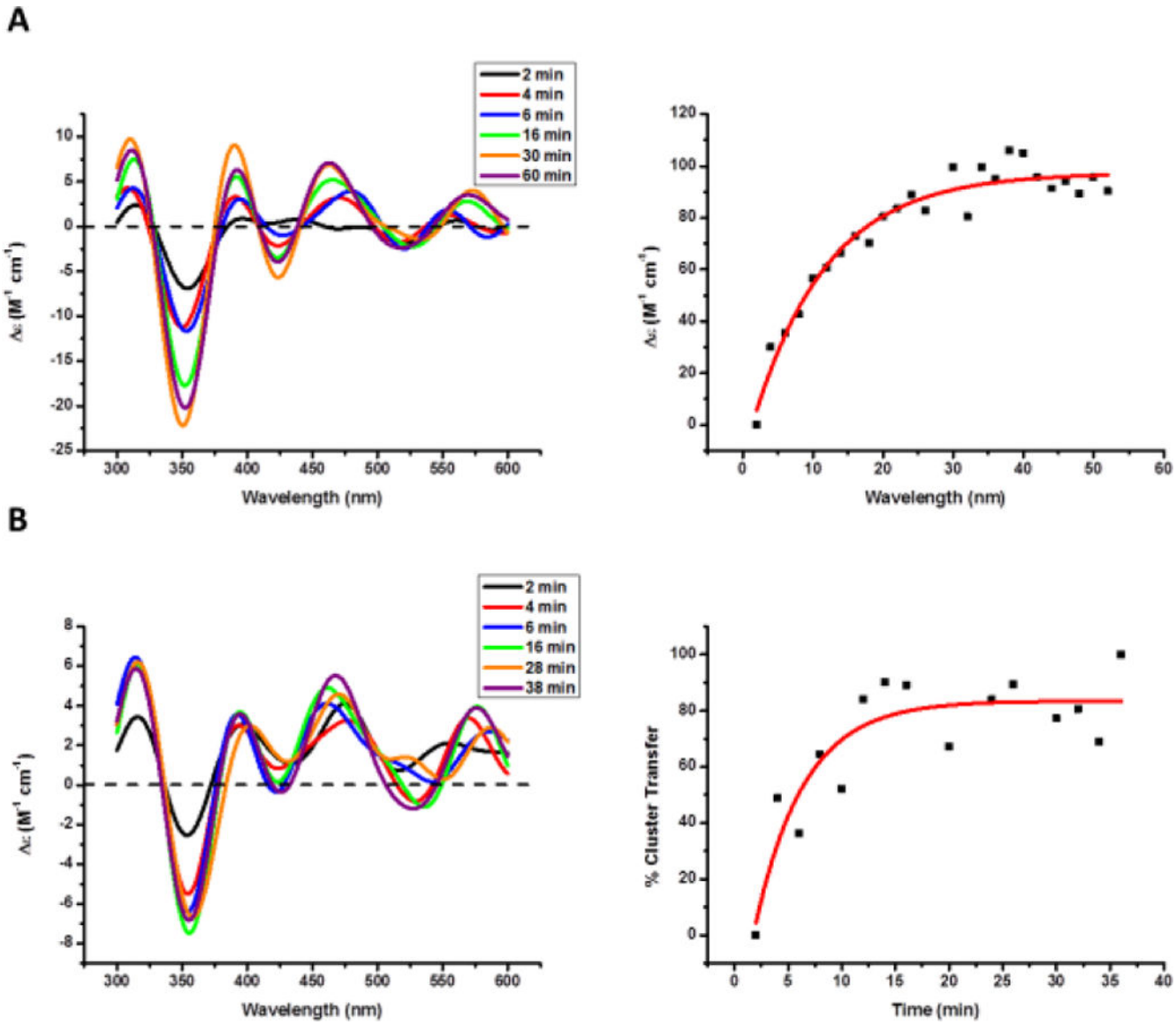

Figure 5.

Time course measurements for cluster transfer from holo ISCU to **(A)** apo BOLA3-Grx3 and **(B)** apo BOLA3-GLRX5 under anaerobic conditions. The CD signal intensity was monitored at room temperature for a 1:1 donor:acceptor cluster stoichiometry in the presence of 3 mM GSH. CD spectra in the 300–600 nm region were recorded every 2 min.

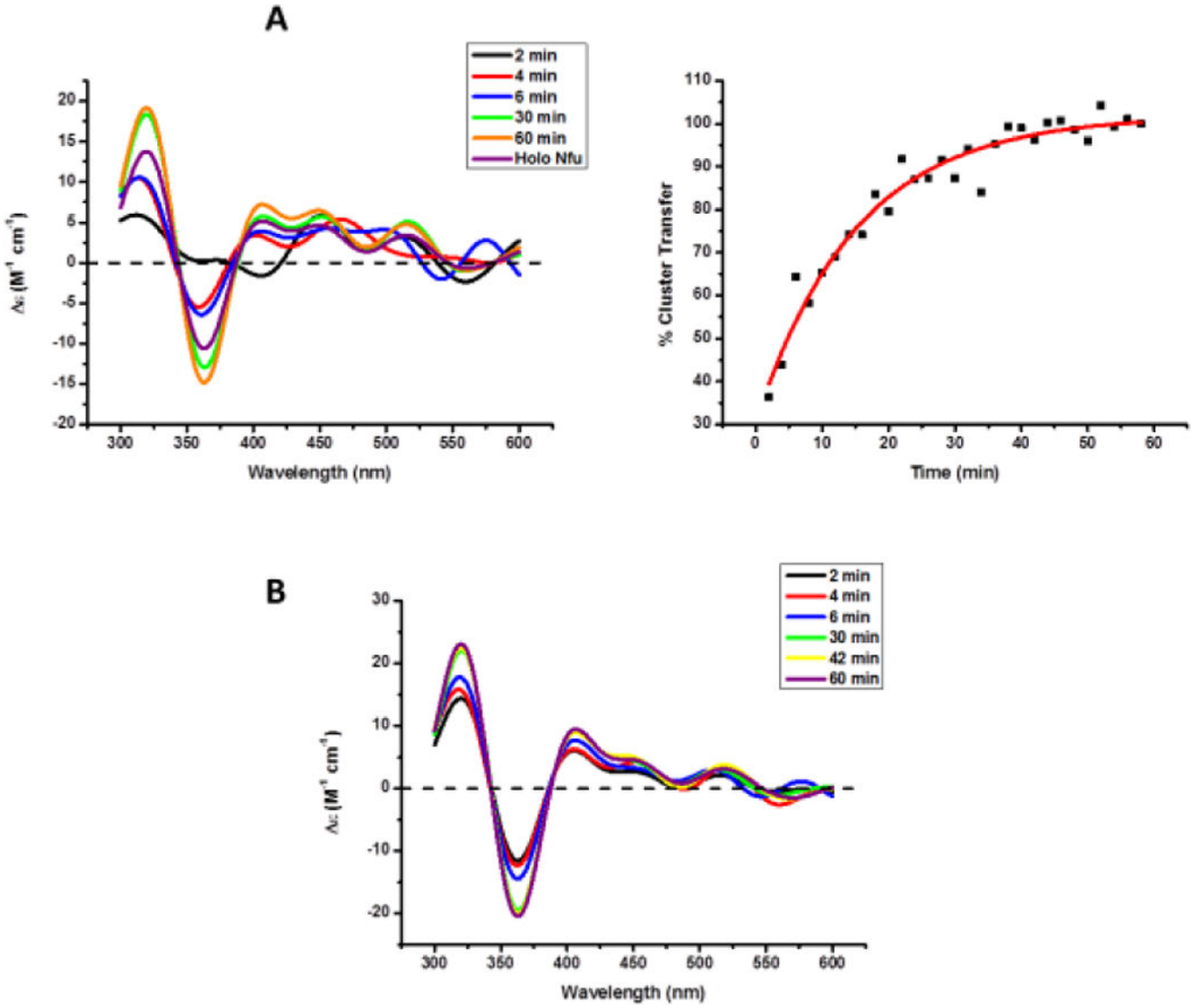

Figure 6.

(A) Time course measurements for the cluster transfer experiment from holo BOLA3 to apo NFU1 under anaerobic conditions. The CD response was monitored at room temperature for a 1:1 donor:acceptor cluster stoichiometry. CD spectra were collected in the 300–600 nm range every 2 min. **(B)** Time course measurements for the cluster transfer experiment from holo NFU1 to apo BOLA3 under anaerobic conditions. The CD response was monitored at room temperature for a 1:1 donor:acceptor cluster stoichiometry. The CD signal does not show any appearance of new features, ruling out formation of any mixed NFU1-BOLA3 species

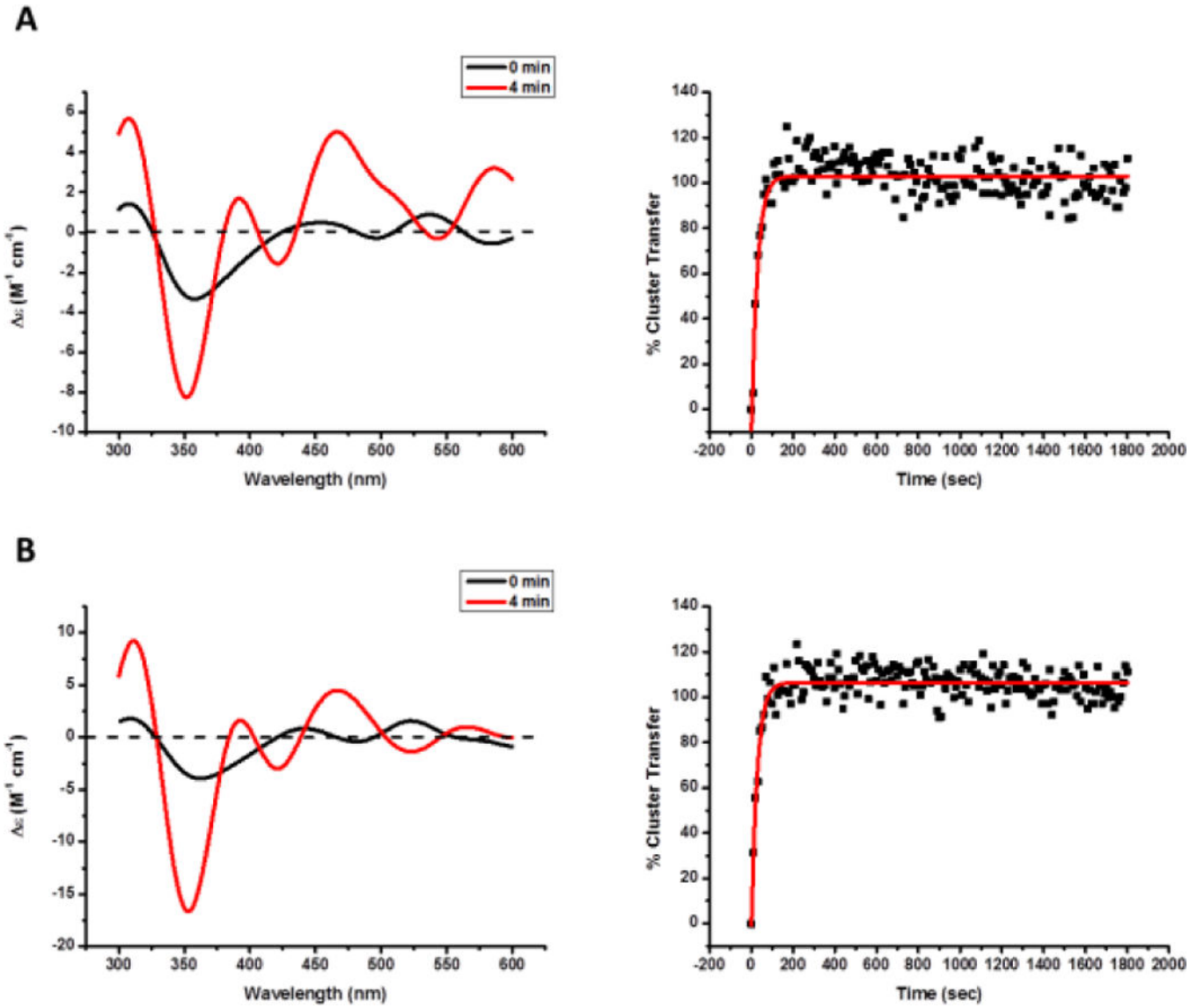

Figure 7.

[2Fe–2S](GS)$_4$ cluster uptake by a 1:1 equimolar mixture of **(A)** BOLA3 and GLRX5 **(B)** BOLA3 and Grx3. Uptake of cluster was followed by monitoring the appearance of a distinct peak at 350 nm in the CD signal for 2:1 cluster:protein stoichiometry under anaerobic conditions.

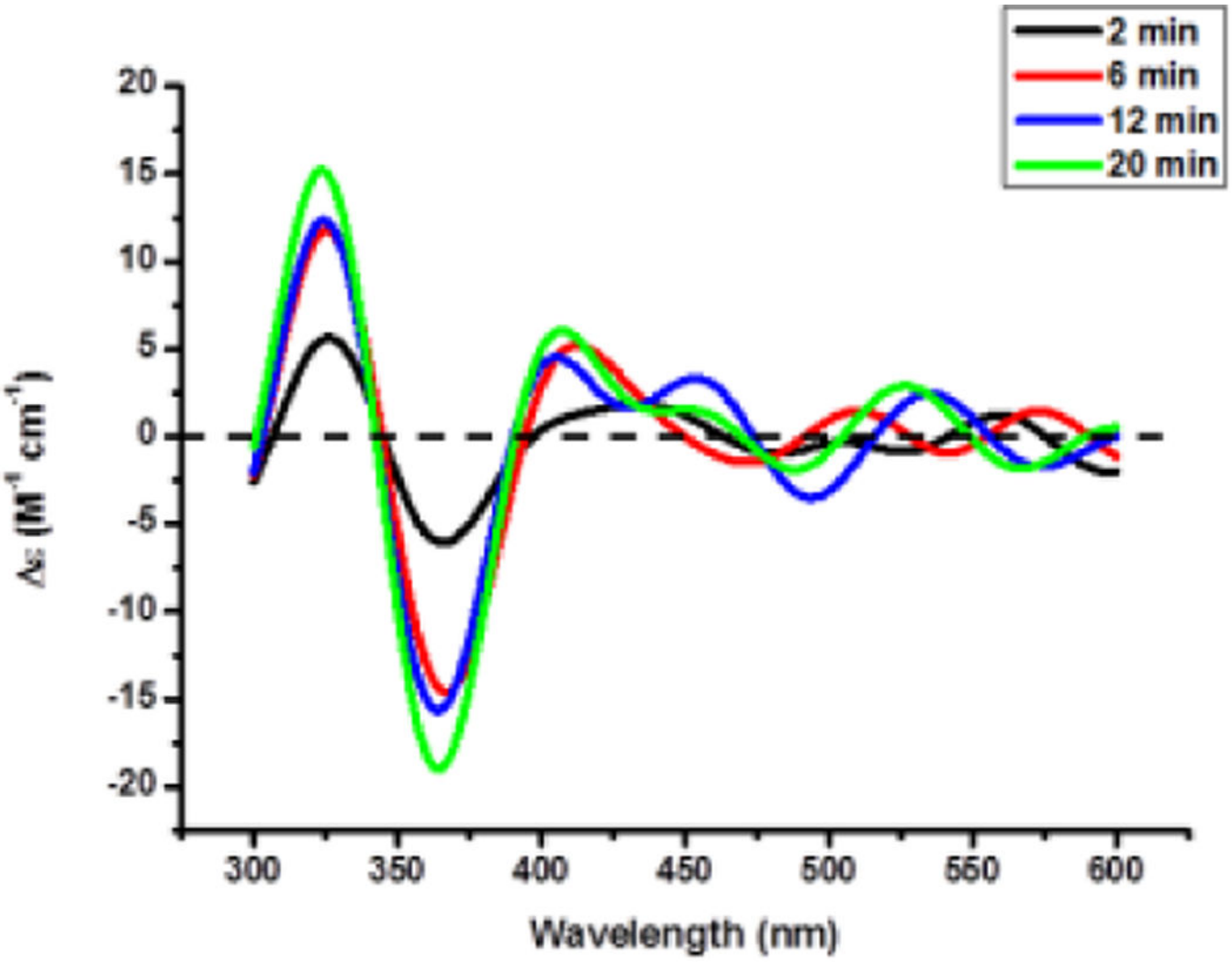

Figure 8.

$[2Fe–2S](GS)_4$ cluster uptake by a 1:1 equimolar mixture of BOLA3 and NFU1 under anaerobic conditions. The final CD signal resembles that of holo NFU1, indicating that the cluster is taken up by NFU1 only and rules out formation of a cluster-bound NFU1-BOLA3 heterocomplex.

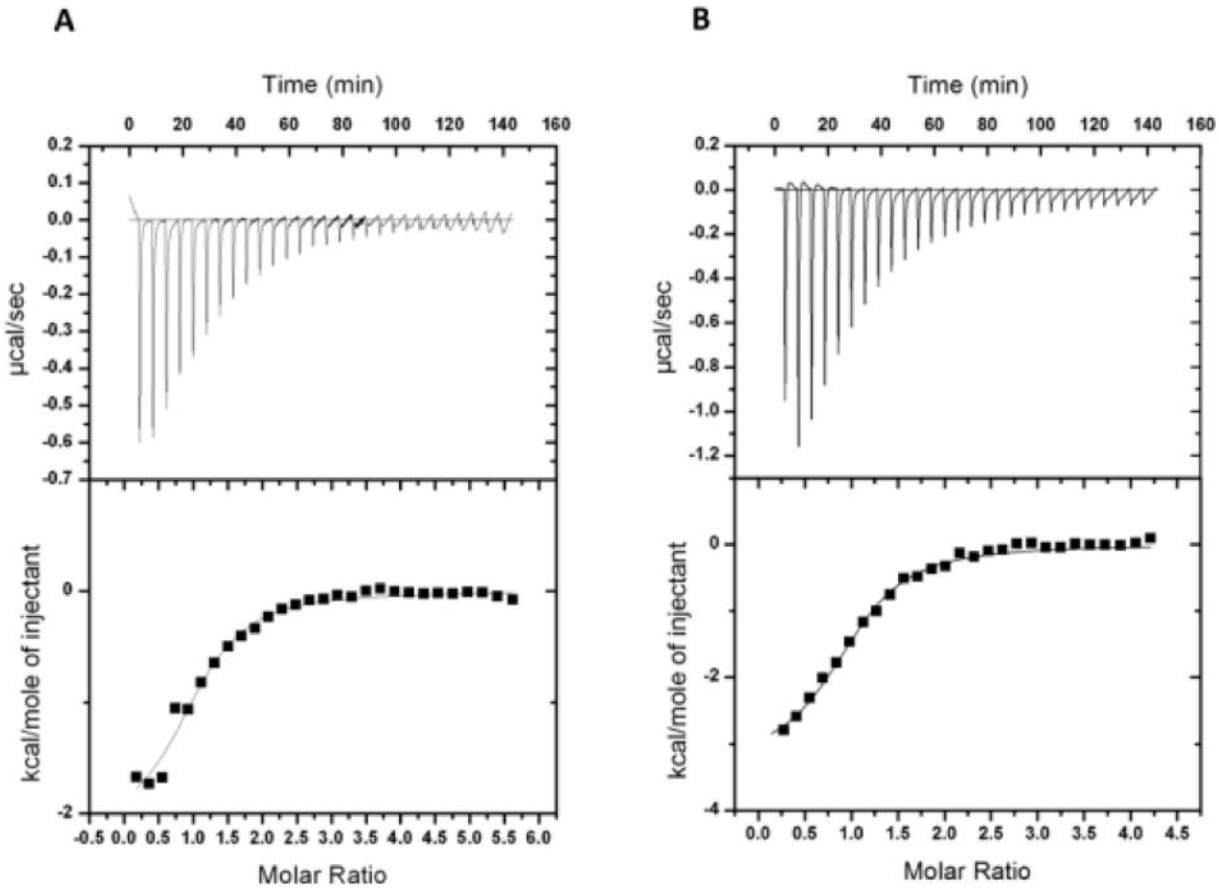

Figure 9.

Isothermal titration calorimetry plots for **(A)** Apo BOLA3 titrated to apo GLRX5 (N= 1.02 ± 0.05, K= 1.2 ×10^5 ± 2.5 ×10^4 M^{-1}, ΔH= −2169 ± 143.1 J mol^{-1}, ΔS= 16 J mol^{-1} K^{-1}). **(B)** Apo BOLA3 titrated to apo Grx3 (N= 0.98 ± 0.03, K= 1.5 ×10^5 ± 2 ×10^4 M^{-1}, ΔH= −3294 ± 121 J mol^{-1}, ΔS= 13 J mol^{-1} K^{-1}). The experiments were performed in the presence of 5 mM GSH in 50 mM HEPES, 100 mM NaCl, pH 7.5. 1 mM BOLA3 was titrated into 50 µM GLRX5/Grx3 in 10 µL aliquots over a period of 24 s with a 5 min interval at 30 °C.

Table 1.

Second-order rate constants for cluster transfer reactions involving BOLA3-GLRX5 and BOLA3-Grx3.

Cluster Transfer	2nd Order Rate Constant(M^{-1} min^{-1})
BOLA3-GLRX5 to FDX2	2800 ± 380
BOLA3-Grx3 to FDX2	2600 ± 540
BOLA3-GLRX5 to FDX1	1400 ± 140
BOLA3-Grx3 to FDX1	1800 ± 170
BOLA3-GLRX5 to NFU1	No Transfer
BOLA3-Grx3 to NFU1	No Transfer
ISCU to BOLA3-GLRX5	14000 ± 1000
ISCU to BOLA3-Grx3	4600 ± 870
[2Fe-2S](GS)$_4$ to BOLA3-GLRX5	28000 ± 2800
[2Fe-2S](GS)4 to BOLA3-Grx3	29000 ± 3000
NFU1 to BOLA3-GLRX5	No Transfer
NFU1 to BOLA3-Grx3	No Transfer