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Heterotrifunctional Chemical Cross-Linking Mass Spectrometry Confirms Physical Interaction between Human Frataxin and ISU

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Supporting Information

ABSTRACT: The progressive neurodegenerative disease Friedreich's ataxia is caused by a decreased level of expression of frataxin, a putative iron chaperone. Frataxin is thought to transiently interact with ISU, the scaffold protein onto which iron−sulfur clusters are assembled, to deliver ferrous iron. Photoreactive heterotrifunctional chemical cross-linking confirmed the interaction between frataxin and ISU in the presence of iron and validated that transient interactions can be covalently trapped with this method. Because frataxin may participate in transient interactions with other mitochondrial proteins, this cross-linking approach may reveal new protein partners and pathways in which it interacts and help deduce direct, downstream consequences of its deficiency.

The neuromuscular degenerative disease Friedreich's ataxia (FA), the most common hereditary ataxia,[1] is most frequently caused by a triplet repeat expansion in the nuclear gene that encodes the mitochondrial protein frataxin.[2] This leads to frataxin deficiency that ultimately causes oxidative stress, respiratory chain dysfunction, and mitochondrial iron accumulation in a nonbioavailable form.[3] A key challenge for FA research is to understand the function of frataxin in biological networks to conduct and/or regulate iron utilization and homeostasis. Studies with the human, yeast, and bacterial systems have indicated that frataxin has specific binding partners in the Fe−S cluster biosynthesis pathway such as the ISU scaffold protein on which Fe−S clusters are assembled,[4−6] the cysteine desulfurase NFS that supplies the sulfur,[7,8] an accessory protein ISD11,[9] and the chaperone HSC20.[10] There is also recent evidence that mammalian frataxin interacts with the preassembled ISU−NFS−ISD11 complex.[11] This has also been confirmed in yeast and *Escherichia coli*. Human and yeast frataxin can form relatively tight, iron-mediated interactions with ISU,[5,6] but this is not the case with the bacterial system where no direct interaction is observed;[8] thus, studies with the human proteins are most relevant to understanding FA. However, frataxin may also be involved in other pathways of iron usage and metabolism,[12] which adds an additional layer of complexity to understanding the molecular basis of FA. Thus far, teasing apart the protein interactions in which frataxin participates has proven to be difficult. More insight into the role of frataxin in heme synthesis, iron storage, and oxidative phosphorylation is needed. Clearly, these must be transient interactions that cannot always be observed by traditional techniques.[13] Here we present the use of photoreactive chemical cross-linking to covalently trap the frataxin−ISU interaction, providing an alternative approach to finding direct, but transient, interacting proteins.

Sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido)-hexanoamido] ethyl-1,3′-dithiopropionate (sulfo-SBED) is a trifunctional cross-linker containing an *N*-hydroxysuccinimide ester group that reacts with lysine amines from the bait protein frataxin, a photoreactive phenyl azide that will cross-link to the interacting prey protein ISU, and a biotin group to aid in detection and/or affinity capture of the cross-linked complexes (Figure 1A). By separating the labeling and photoactivation steps, we initiated covalent cross-linking in a controlled manner just by exposure to long wavelength light. Sulfo-SBED also contains a disulfide bond in the linker so that the cross-link can be reversed with standard reducing agents. Thus, the biotin group from SBED-labeled frataxin is transferred to the

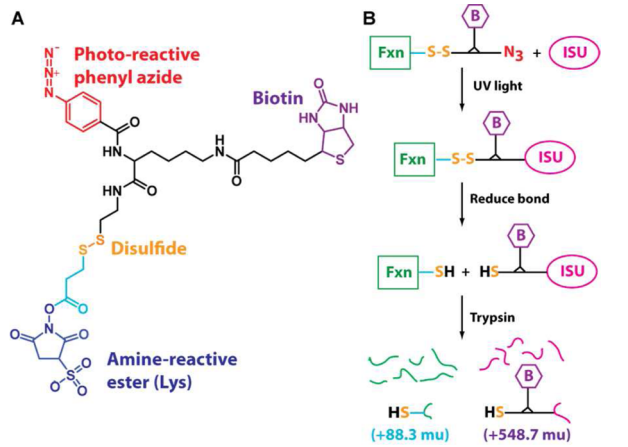

Figure 1. (A) Sulfo-SBED photoreactive cross-linker. (B) Photo-activated cross-linking scheme using SBED-frataxin (Fxn). 3-Mercaptopropanamido-lysines from frataxin peptides have a mass increase of 88.3 mu, while biotinylated ISU after label transfer has a mass increase of 548.7 mu.

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interacting protein (Figure 1B), which can then be detected via a chromogenic Western blot by streptavidin.

Purified human frataxin (mature form, residues 81−210) was reacted with sulfo-SBED in the dark. SBED-frataxin was then incubated with and without human Fe^{2+}-ISU (mature form, residues 35−167) in an anaerobic glovebox to maintain reduced cysteine thiolates and ferrous iron, followed by UV activation at 365 nm. Samples were run on a sodium dodecyl sulfate−polyacrylamide gel, transferred to a membrane, and probed with a streptavidin−alkaline phosphatase conjugate to colorimetrically detect the presence of a biotin label (Figure 2A). The blot indicates that purified frataxin (~14 kDa) was successfully labeled by sulfo-SBED (lane 2), which is reversible with dithiothreitol (DTT) (lane 3). As a control for UV activation, no covalent cross-link was observed for SBED-frataxin and ISU without UV exposure (lane 5). However, a new biotinylated protein band at ~29 kDa is only present when SBED-frataxin and Fe^{2+}-ISU were exposed to UV light, indicating a cross-linked complex was formed (lane 6). This band disappeared with DTT reduction, confirming that this cross-link was present (lane 7). A small amount of cross-linking also occurred with SBED-frataxin (lane 8), and this also can be reversed by DTT (lane 9). It is known that frataxin can oligomerize and/or aggregate, so the presence of a small amount of intermolecular cross-linking was not unexpected.[14] We also tested for nonspecific cross-linking using bovine serum albumin (BSA) at the same concentration as ISU. Western analysis did not reveal a significant frataxin−BSA cross-linked complex (83 kDa) after UV activation (Figure S1 of the Supporting Information). This confirms that the biotinylated band observed for the frataxin−ISU cross-linked sample is specific to these two proteins.

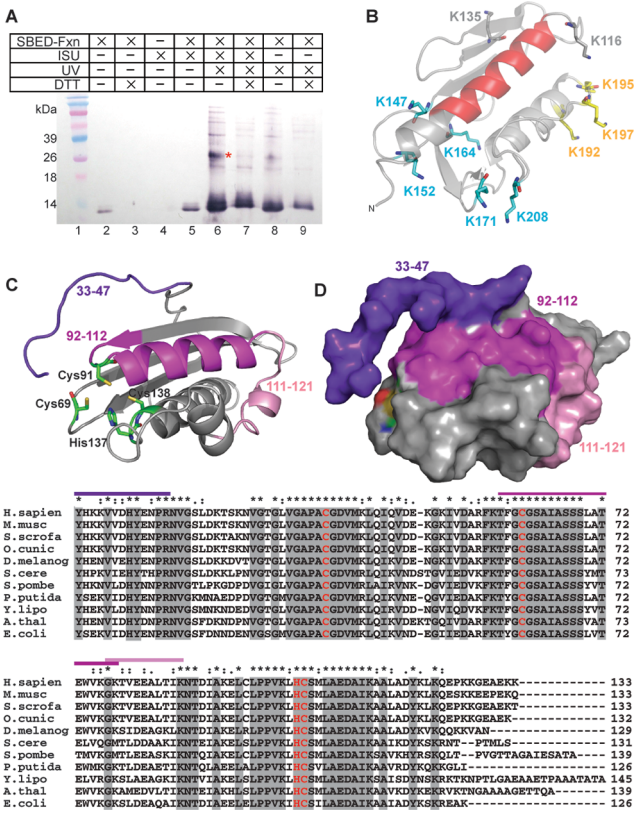

Figure 2. Photoactivated chemical cross-linking of frataxin and ISU. (A) Western blot of cross-linking reactions and controls using the streptavidin−alkaline phosphatase conjugate to detect the biotin tag from SBED. The ~29 kDa frataxin−ISU cross-linked band is indicated with a red asterisk. A detailed figure legend can be found in the Supporting Information. (B) Location of lysine residues that reacted with sulfo-SBED mapped to the structure of frataxin (Protein Data Bank entry 1LY7)[15] with labeled lysines colored cyan, unlabeled lysines colored yellow, and no data colored gray. The acidic α_1-helix is colored red. (C) ISU peptides involved in a cross-link with frataxin mapped to the mouse ISU homologue structure (Protein Data Bank entry 1WFZ). (D) Surface rendering of panel C. (E) ClustalX[16] sequence alignment of various ISU homologues. Identical residues are indicated with asterisks, with highly and moderately conserved residues marked with semicolons and dots, respectively. The [2Fe-2S] coordinating residues are colored red. Cross-linked ISU peptides are indicated above the sequence in purple, magenta, and pink.

To identify the proteins present, the cross-linked band from the frataxin−ISU sample was excised from the gel, reduced with DTT, treated with iodoacetamide, and then digested with trypsin. The samples were subjected to matrix-assisted laser desorption ionization time of flight mass spectrometry to identify peptides involved in the cross-links (Figure S2 and Tables S1 and S2 of the Supporting Information).[17] Five of 10 frataxin Lys residues contained the 3-mercaptopropanamido moiety of the SBED label left over from reduction of the disulfide in the linker (Figure 2B, cyan). No SBED label was detected for three Lys residues despite identification of the trypsin peptides containing those unmodified residues (Figure 2B, yellow). Two Lys residues could not be detected from the digest (Figure 2B, gray). This is due to a missed trypsin cleavage site from the presence of a proline residue following K116, which resulted in a peptide outside the MS mass range (Figure S2 of the Supporting Information). Thus, it is unclear if Lys116 and Lys135 were actually labeled by SBED. Because the disulfide bond in the linker between frataxin and ISU was reduced with DTT, it is not possible to determine whether labeled Lys residues were covalently cross-linked to ISU, because breaking the disulfide bond with or without a bound protein results in the same mass increase (Figure 1B). It is possible to perform the trypsin digest on the intact cross-linked complex without reduction of the linker disulfide. However, this would add much more complexity to the mass spectra analysis because two peptides from two proteins will be cross-linked together, but even from our data, it is clear that frataxin lysines modified with SBED tend to cluster around the acidic α_1-helix that is proposed to be involved in iron coordination or binding with ISU.[6,11] A recent report using mutagenesis and affinity tag pull-down assays showed that E108, E111, D124, N146, and W155 are essential for the ISU interaction.[11] Our cross-linking identified lysines close to these residues as being modified and potentially cross-linked to ISU (K147, K152, and K164). It is also significant to note that modification of frataxin lysines by SBED did not prohibit the frataxin−ISU complex from forming, which is an important factor in the success of chemical cross-linking identifying other potential interactors.

For ISU, five peptides contain the transferred biotin label after disulfide reduction of the frataxin−ISU bond (Table S2 and Figure S2 of the Supporting Information). Figure 2C shows the location of these peptides on the structure of a highly homologous ISU protein from mouse. Three of these peptides (residues 33−38, 38−47, and 39−47) are at the disordered N-terminus; one peptide is near the putative Fe−S cluster-coordinating cysteines (residues 99−112), and the last is adjacent to the segment of residues 99−112 (residues 111−

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121). Chemical cross-linking reveals that the ISU peptides form a surface that surrounds the three highly conserved cysteines (Cys69, Cys91, and Cys138) and one histidine (His137) that coordinate the Fe−S cluster during assembly based on homologous protein structures (Figure 2D).[18] It is likely, given the 14 Å length of the cross-linker, that these ISU peptides comprise the binding site for frataxin, which was previously uncharacterized. One of these cross-linked peptides actually contains Cys91, a Fe−S cluster-coordinating residue.

An amino acid sequence alignment of ISU proteins reveals significant conservation of residues within the peptide regions that cross-linked to frataxin (Figure 2E). Our results indicate that the N-terminus of ISU is involved in frataxin binding. This disordered domain extends over the Fe−S cluster assembly site and is unique to eukaryotic frataxin proteins. There are several conserved, positively charged side chains within the segment of residues 33−47, which could interact specifically with the acidic, iron-binding α_1-helix of frataxin. Prokaryotic IscU proteins lack this N-terminal extension, and there is speculation that this is why an interaction between *E. coli* frataxin (CyaY) and IscU has not been observed,[8] in contrast to eukaryotic proteins. Given that formation of the frataxin−ISU complex is unique to eukaryotes and probably transient, the cross-linking results presented provide key structural information about the nature of the interaction, which is especially important for the human proteins where the ISU interaction surface has yet to be defined. The chemistry of cluster formation of the ISU proteins is far from being understood. We provide new insight into the previously unknown surface on ISU flanking the Fe−S cluster assembly site that associates with frataxin, presumably for iron transfer.

We anticipate that photoactivated chemical cross-linking can potentially build an interaction "map" for the proteins of the Fe−S cluster assembly pathway in humans, to improve our understanding of the role frataxin plays in this vital process. Because frataxin may also participate in transient interactions with other mitochondrial proteins, this cross-linking approach has the power to reveal new interacting proteins as part of our ongoing proteomic studies. Thus, via identification of new interactions, more information will be gleaned about iron homeostatic pathways and how they relate to FA pathophysiology.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, expanded Figure 2 legend, Figures S1 and S2, and Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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H.M.W., L.E.G., A.P.A., and Y.W. performed and analyzed the experimental work. L.S.B. wrote the manuscript with contributions of all authors.

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Notes

The authors declare no competing financial interests.

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