Protein-Interaction Affinity Gradient Drives [4Fe–4S] Cluster Insertion in Human Lipoyl Synthase

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ABSTRACT: Human lipoyl synthase (LIAS) is an enzyme containing two [4Fe–4S] clusters (named FeSRS and FeSaux) involved in the biosynthesis of the lipoyl cofactor. The mechanism by which a [4Fe–4S] cluster is inserted into LIAS has thus far remained elusive. Here we show that NFU1 and ISCA1 of the mitochondrial iron–sulfur cluster assembly machinery, via forming a heterodimeric complex, are the key factors for the insertion of a [4Fe–4S] cluster into the FeSRS site of LIAS. In this process, the crucial actor is the C-domain of NFU1, which, by exploiting a protein-interaction affinity gradient increasing from ISCA1 to LIAS, drives the cluster to its final destination.

By mapping these changes on the structure of the C-terminal domain of apo NFU1, we observed that the two helices of the C-terminal domain of apo NFU1 are significantly affected by the protein–protein interaction, while the β-sheet is essentially unaffected. The cluster-binding CXXC motif of apo NFU1, enclosed between the two helices, is also involved in the interaction with AI LIAS, indicating that AI LIAS in the complex with NFU1 is positioned close to the cluster-binding region.

Complex formation was also followed by performing in parallel 1H-15N HSQC spectra and analytical gel filtrations on protein mixtures obtained by adding one or more equivalents of 15N-apo NFU1 to unlabeled AI LIAS (see the Supporting Information for details). At a 1:1 protein ratio, apo NFU1 is fully complexed with AI LIAS, as no signal of isolated apo NFU1 is present in the NMR spectrum (Figure 1B). The cluster-binding CXXC motif of apo NFU1, enclosed between the two helices, is also involved in the interaction with AI LIAS, indicating that AI LIAS in the complex with NFU1 is positioned close to the cluster-binding region.

As the first step, we have investigated, by nuclear magnetic resonance (NMR) and analytical gel filtration, the interaction between apo NFU1 and as-isolated LIAS (AI LIAS, hereafter), which contains a [4Fe–4S] cluster bound mostly at the FeSaux site (see the Experimental Section in the Supporting Information for details, Table S1 and Figure S1). In the analytical gel filtration chromatogram of a 1:1 apo NFU1–AI LIAS mixture, a main peak containing both proteins is present with an elution volume smaller than that of the two isolated proteins (Figure S2). The elution volume of this peak is consistent with the presence of a heterodimeric complex, which is the predominant form at the 1:1 apo NFU1–AI LIAS ratio. We also observed that, when apo 15N-NFU1 is stepwise titrated with AI LIAS up to a 1:1 protein ratio, chemical shift changes occurred in the 1H-15N heteronuclear single quantum coherence (HSQC) maps of apo NFU1 in intermediate/slow exchange regimes on the NMR time scale, indicating the occurrence of the apo NFU1–AI LIAS interaction (Figures 1A and S3). The majority of the affected residues are located in the C-domain of NFU1 (see Figures S3 and S4 for details), thus revealing the C-domain of NFU1 as the crucial player driving the NFU1–AI LIAS interaction. These data are in agreement with previous yeast-two-hybrid assay studies.

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NFU1 (Figure 2). Upon addition of three and four equivalents of apo NFU1, the NMR signal of isolated apo NFU1 increases in intensity and concomitantly, in the chromatogram of the analytical gel filtration, the peaks of monomeric and dimeric isolated apo NFU1 gradually increase their intensity with respect to the peak of the heterodimeric complex (Figure 2). In fraction 2 of the sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2), we can consistently observe the increase of the intensity of the NFU1 band with respect to that of AI LIAS along the additions of apo NFU1.

As the following step, unlabeled AI LIAS was stepwise added to the apo ISCA1–15N-NFU1 complex obtained as previously described.13 The overlay of the 1H-15N HSQC maps of the two individual apo unlabeled ISCA1–15N-NFU1 and apo 15N-NFU1–unlabeled AI LIAS complexes clearly shows that the spectra of these two complexes are different (Figure S5) and thus they can be exploited to monitor a possible conversion between the two complexes. Upon addition of one equivalent of AI LIAS, several NMR signals of 15N-NFU1 broaden beyond detection or change their chemical shifts (Figure 3A), indicating that apo NFU1 changes its interactions pattern. When this spectrum is compared with that of heterodimeric complex between apo 15N-NFU1 and unlabeled AI LIAS (Figure 3B), it results that the two spectra are well superimposable, indicating that apo NFU1 is preferentially interacting with AI LIAS to form the apo NFU1–AI LIAS heterodimeric complex. Consistently, no NMR signals of free apo 15N-NFU1 are observed (compare black spectrum in Figure 3A with green spectrum in Figure 3B), indicating that NFU1 remains in a complexed form. The analytical gel filtration of the final mixture showed an intense peak with an elution volume smaller than those of the three isolated monomeric proteins and of the heterodimeric ISCA1–NFU1 complex (Figure 3C and Figure S2), consistent with the formation of the higher molecular weight apo NFU1–AI LIAS dimeric complex. Furthermore, a low-intensity peak eluting at 17.7 mL is formed upon addition of AI LIAS to the apo ISCA1–NFU1 complex (Figure 3C) whose elution volume matches with that of monomeric apo ISCA1, thus indicating that ISCA1 is released in solution as a free protein. In conclusion, NMR and analytical gel filtration data allow to exclude the formation of a heterotrimeric ISCA1-NFU1-AI LIAS complex and show that AI LIAS displaces ISCA1 from the heterodimeric apo ISCA1-NFU1 complex to form a heterodimeric complex with NFU1.

Figure 1. Apo NFU1 interacts with AI LIAS via its C-terminal domain. (A) Overlay of 1H-15N HSQC maps of 15N-apo NFU1 (black) and the 1:1 15N-apo NFU1-unlabeled AI LIAS mixture (red).(B) Meaningful chemical shift changes are shown in green on the structure of the C-domain of apo NFU1.

Figure 2. Apo NFU1 and AI LIAS form a heterodimeric complex. On the left, 1H-15N HSQC maps at different NFU1–AI LIAS ratios enclosing the signal of Arg 96 of 15N-NFU1 in slow exchange regime on the NMR time scale upon NFU1–AI LIAS complex formation. On the right, analytical gel filtration chromatograms of the same mixtures analyzed by NMR. SDS-PAGE of fraction 1 (eluted between 16.0 and 16.5 mL) and that of fraction 2 (eluted at 16.5–17.0 mL) are reported on the right of each chromatogram.
physiological candidate, although it may not be the only possibility,\textsuperscript{15,16} to insert a [4Fe–4S]\textsuperscript{2+} cluster into LIAS.\textsuperscript{1H-15N} HSQC experiments titrating the [4Fe–4S]\textsuperscript{2+} unlabeled ISCA1−15N-NFU1 complex with unlabeled AI LIAS up to a 1:1 ratio were then performed. Some NMR signals allowed us to monitor the cluster release from complexed 15N-NFU1, as their chemical shifts exclusively depend on the presence ([4Fe–4S] in Figure 4A,B) or absence (apo in Figure 4A,B) of the [4Fe–4S]\textsuperscript{2+} cluster in NFU1 complexed with either unlabeled AI LIAS or ISCA1 (Figures S5 and S6). In the final mixture of the titration, these signals of complexed 15N-NFU1 overlay with those corresponding to the formation of apo complexed 15N-NFU1 (Figure 4A,B), thus indicating that the [4Fe–4S]\textsuperscript{2+} cluster is no longer bound to NFU1. The 1H-15N HSQC spectra also showed that the signals of the final 1:1 mixture overlap with those of the apo 15N-NFU1−AI LIAS complex and not with those of the apo ISCA1−15N-NFU1 complex (Figure S7), indicating the formation of the apo state of NFU1 complexed with LIAS. Thus, the displacement of ISCA1 from the [4Fe–4S]\textsuperscript{2+} ISCA1−NFU1 complex to form a dimeric complex between NFU1 and LIAS occurs similarly to what was observed in the absence of cluster transfer (Figure 3).

Figure 3. AI LIAS displaces ISCA1 from the apo ISCA1−NFU1 complex to form a heterodimeric complex with apo NFU1. (A,B) Overlay of 1H-15N HSQC maps of 15N-apo NFU1 in different states indicated by color codes. (C) Analytical gel filtration chromatograms of apo ISCA1 (violet), apo NFU1 (black), apo ISCA1−NFU1 complex (red), and a 1:1 mixture between apo ISCA1−NFU1 complex and AI LIAS (green).

Figure 4. [4Fe–4S]\textsuperscript{2+} ISCA1−NFU1 transfers the cluster to the FeS\textsubscript{38} site of AI LIAS. (A and B) Overlay of two different regions of 1H-15N HSQC maps of [4Fe–4S]\textsuperscript{2+} ISCA1−NFU1 complex (black), apo NFU1−AI LIAS complex (blue) and of a 1:1 mixture of the [4Fe–4S]\textsuperscript{2+} ISCA1−NFU1 complex and AI LIAS (red) (NFU1 is 15N-labeled, ISCA1 and AI LIAS are unlabeled). (C) Paramagnetic 1D 1H NMR spectra of (I) a 1:1 mixture of [4Fe–4S]\textsuperscript{2+} ISCA1−NFU1 and C106/C111/C117A AI LIAS, obtained by anaerobically mixing the two proteins, (II) C106/C111/C117A AI LIAS and (III). In the inset of panel I, a far-shifted region of the paramagnetic NMR spectrum is shown at 298 K (black) and 290 K (red).
We also followed cluster insertion into LIAS by paramagnetic 1D 1H NMR. A triple C106/C111/C117A LIAS variant was used as cluster acceptor because this variant lacks the cysteine ligands of Fe₄S₄ and can allow exclusively monitoring cluster insertion into Fe₄S₄. C106/C111/C117A A1 LIAS was purified with ~30% of [4Fe–4S] clusters bound to the Fe₄S₄ site (Table S1). Upon addition of one equivalent of the [4Fe–4S]²⁺ ISCA1–NFU1 complex to the C106/C111/C117A A1 LIAS variant, the intensities of the ¹H NMR signals at 15–11 ppm, assigned to βCH₃ of the ligands of Fe₄S₄ increase in intensity (Figure 4C), indicating that cluster insertion into the Fe₄S₄ site occurred, thus being the cluster not degraded or released in solution.

The anti-Curie temperature dependence and the chemical shift values of these signals are consistent with an oxidized [4Fe–4S]²⁺ cluster bound to LIAS, in agreement with the UV–visible spectrum of the final 1:1 mixture (Figure S8). In addition, we observed two other signals in the 46–36 ppm region with Curie temperature dependence (inset of Figure 4C). Their temperature dependence and chemical shifts are typical of protons of cysteine residues bound to a reduced [4Fe–4S]²⁻ cluster,¹¹ thus indicating that a fraction of Fe₄S₄ is in the reduced state. The presence of cysteine residues with chemical shifts typical of both reduced and oxidized [4Fe–4S] clusters suggests that Fe₄S₄ can be partially reduced by 5 mM dithiothreitol (DTT), the only reductant present in the mixture. This result is in agreement with what previously observed in wild-type LIAS,³ is fully consistent with the electron transfer function of Fe₄S₄ in the catalytic mechanism³⁻¹⁰ as well as with a reduction potential of Fe₄S₄ lower than that of DTT, as typically observed for radical SAM [4Fe–4S] clusters.¹⁰

In conclusion, we have shown that the C-domain of NFU1 is the triggering factor for the insertion of a [4Fe–4S] cluster into the Fe₄S₄ site of LIAS thanks to its specific interaction with LIAS. The strength of this interaction displaces ISCA1, which is the scaffold protein, and its ability to assemble a [4Fe–4S] cluster. The C-domain of NFU1 results a stronger interacting partner of LIAS than ISCA1. In the mitochondrial ISC assembly machinery, the C-domain drives first [4Fe–4S] cluster delivery from the ISCA1–ISCA2 complex, where the [4Fe–4S]²⁺ cluster is assembled,¹³ to the [4Fe–4S]²⁺ ISCA1–NFU1 intermediate complex,¹³ which then specifically directs the cluster into the Fe₄S₄ site of LIAS. These sequential molecular events are driven by an interaction affinity gradient of the C-domain of NFU1 increasing from ISCA1 to LIAS. Our data do not exclude that a dimeric [4Fe–4S]²⁺ NFU1-dependent pathway might be present in human cells, as proposed in yeast,¹⁵ as an alternative pathway.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge from the [link](https://pubs.acs.org/10.1021/jacs.1c13626). Detailed experimental procedures (PDF)

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#### Notes

The authors declare no competing financial interest.

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