Does the CX_2CX_2C Motif Coordinate the [4Fe-4S] Cluster of NadA

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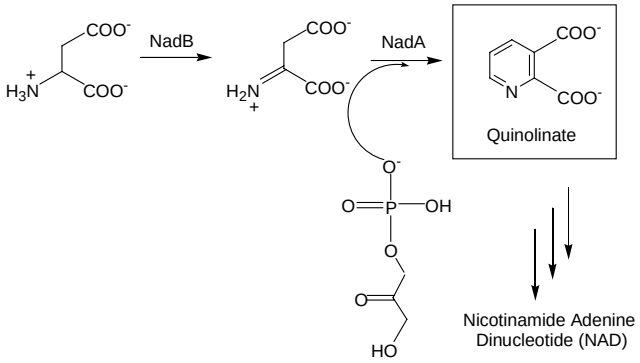
Nicotinamide adenine dinucleotide (NAD) is a vital enzymatic cofactor and is present in all living organisms. [1] NAD functions as a hydride acceptor resulting in the formation of its reduced form NADH, which is a hydride donor in redox reactions. In *Escherichia coli* (*E. coli*) and most other prokaryotes, NAD is produced from quinolinic acid, derived from the action of two enzymes, L-aspartate oxidase (NadB) and quinolinate synthetase (NadA) (Scheme 1). Recently, the Booker laboratory has shown that NadA harbors a [4Fe-4S] cluster. NadA contains nine cysteine residues, three of which lie in a motif (CXXCXXC) that is characteristic of cysteines that ligate iron-sulfur (Fe-S) clusters. Through expression of the *nadA* gene from *E. coli*, and purification and characterization of its gene product, lab the Booker lab intends to determine whether or not the cysteines in this motif coordinate the Fe-S cluster. Purification of the NadA protein was performed anaerobically due to the oxygen sensitivity of the cluster. Five variant NadA proteins have been generated, each of which contains a cysteine to serine substitution. With the removal of each of the cysteines, it is expected that formation of the Fe-S cluster will be impeded, resulting in an inactive NadA protein. The variant proteins will then be characterized by iron analysis and UV-Vis spectroscopy.

Introduction

Nicotinamide adenine dinucleotide (NAD) is a vital enzymatic cofactor that is present in all living organisms. NAD functions as a hydride acceptor resulting in the formation of its reduced form NADH, which is a hydride donor in redox reactions. Quinolinic acid, a precursor of NAD, is synthesized in *E. coli* by condensation of L-aspartate and dihydroxyacetone phosphate (DHAP) in an anaerobic pathway [2,3] (Scheme 1). This reaction requires two essential enzymes, L-aspartate oxidase (NadB) and quinolinate synthetase (NadA). The *nadA* gene has been cloned and sequenced, however little work has been published describing the mechanism of NadA because of its instability. The NadA protein harbors an oxygen sensitive iron-sulfur (4Fe-4S) cluster, which has hampered many attempts to purify it.[4]



Scheme 1 The biosynthetic pathway of NAD in *E. coli.*

Three cysteines are believed to coordinate the Fe-S cluster, and in *E. coli* it has been suggested that the cysteines lie in a CX_2CX_2C motif, which is known to involve iron-sulfur cluster coordination in other iron- sulfur proteins [5]. Research done in the Booker lab has verified that a [4Fe-4S] cluster is present in NadA of *E. coli* [3]**;** however, the cysteines that coordinate the cluster have not been established. To assess whether or not cysteines in the CX_2CX_2C motif coordinate the cluster, we initially carried out an exhaustive sequence alignment of the NadA protein from over 150 species. We found that only three cysteines were conserved throughout all species, only one of which lies in the CX_2CX_2C motif (Scheme 1). To verify that the conserved cysteines were those that ligand the cluster, we generated Cys to Ser variants of each of the cysteines, and isolated the corresponding proteins to investigate their role in the ligation of the [4Fe-4S] cluster.

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Escherichia      MSVMFDPDTAIYPFPPKPTPLSIDEKAYYREKIKRLLKERNAVMVAHYYTDPEIQQLAEE 60
Shigella         MSVMFDPDTAIYPFPPKPTPLSIDEKAYYREKIKRLLKERNAVMVAHYYTDPEIQQLAEE 60
Yersinia         MSEIFDVNAAIYPFPARPVPLDTNEKAFYREKIKTLLKQRDAVLVAHYYTDPEIQALAEE 60
Erwinia          MNILFDSNETIYPFPPKPRPLSVDAKQHYRSRIKTLLRERNAVMVAHYYTDPEIQALAEE 60
                 *. :** : :*****.:* **. : * .**.:** **::*:**:*********** ****
Escherichia      TGGCISDSLEMARFGAKHPASTLLVAGVRFMGETAKILSPEKTILMPTLQAECSLDLGCP 120
Shigella         TGGCISDSLEMARFGAKHPASTLLVAGVRFMGETAKILSPEKTILMPTLQVECSLDLGCP 120
Yersinia         TGGCVADSLEMARFGNNHPASTLLVAGVRFMGETAKILNPEKKVLMPTLNAECSLDLGCP 120
Erwinia          TGGCVADSLEMARFGSTHSASTLLVAGVRFMGETAKILNPEKTILMPTLEAECSLDLGCP 120
                 ****::********* .*.******************.***.:*****:.********
Escherichia      VEEFNAFCDAHPDRTVVVYANTSAAVKARADWVVTSSIAVELIDHLDSLGEKIIWAPDKH 180
Shigella         VEEFNAFYDAHPDRTVVVYANTSAAVKARADWVVTSSIAVELIDHLDSLGEKIIWAPDKH 180
Yersinia         VDEFTAFCDSHPDRTVVVYANTSAAVKAKADWVVTSSIAVELIEHLDSLGEKIIWAPDRH 180
Erwinia          IDAFSRFCDAHPDRTVVVYANTSAAVKARADWVVTSSIAVELIEHLDSLGEKIIWAPDRH 180
                 :: *. * *:******************:**************:**************:*
Escherichia      LGRYVQKQTGGDILCWQGACIVHDEFKTQALTRLQEEYPDAAILVHPESPQAIVDMADAV 240
Shigella         LGRYVQKQTGGDILCWQGACIVHDEFKTQALTRLQEEYPDAAILVHPESPQAIVDMADAV 240
Yersinia         LGSYVQKKSGADVLCWQGACIVHDEFKTQALARMKALYPDAAVLVHPESPQAVVDMADAV 240
Erwinia          LGSYVQKQTGADVLCWQGACIVHDEFKTQALQRMKILYPDAAILVHPESPQSVVEMADAV 240
                 ** ****::*.*:****************** *::  *****:********::*:*****
Escherichia      GSTSQLIAAAKTLPHQRLIVATDRGIFYKMQQAVPDKELLEAPTAGEGATCRSCAHCPWM 300
Shigella         GSTSQLIAAAKALPHQRLIVATDRGIFYKMQQAVPDKELLEAPTAGEGATCRSCAHCPWM 300
Yersinia         GSTSQLIQAAKTLPQKTLIVATDRGIFYKMQQACPDKELFEAPTAGEGATCRSCAHCPWM 300
Erwinia          GSTSQLIQAAKTLPQRELIVATDRGIFYKMQQACPEKTLLEAPTAGEGATCRSCAHCPWM 300
                 ******* ***:**:: ********************* *:* *:*********************
Escherichia      AMNGLQAIAEALEQEGSNHEVHVDERLRERALVPLNRMLDFAATLRG------ 347
Shigella         AMNDLQAIAEALEQEGSNHEVHVDERLRERALVPLNRMLDFAATLRG------ 347
Yersinia         AMNGLRAIAEGLEQGGVMHEIHVDEELRQQALIPLNRMLDFANQLKLQVKGNA 353
Erwinia          AMNGLEAIANGLEQGGHAHEIHVDAALREGALIPLNRMLDFAASLKLRVKGNA 353
                              ***.*.***:.*** * **:*** **: **:********* *:
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Figure 1. Primary sequence alignment of the NadA protein from *Escherichia coli, Shigella flexneri, Yersinia pestis, Erwinia carotovora.* * = conserved amino acid residues
The highlighted residues denote the conserved cysteines that were changed to serine.

Materials and Methods

Cloning NadA gene from E. coli

Loretta Tu, a technician in the Booker lab, generated the C113S, C200S, C291S, C294S, and C297S substitutions in the nadA gene. The genes were cloned into a pET-28a vector such that the gene products would contain a hexahistidine appendage at the N-terminus to facilitate purification by immobilized metal affinity chromatography (IMAC).

Growth and expression of NadA

The C_{297} *nadA* mutant strain was streaked onto a Luria Bertani (LB) plate containing 50 μg mL^{-1} of kanamycin (kan) and 100 μg mL^{-1} of ampicilin (amp). The plate was then grown overnight at 37^0C. A single colony from this plate was used to inoculate 200 mL of LB containing 50 μg mL^{-1} of kan and 100 μg mL^{-1} of amp. The starter culture was grown at 37^OC for 10-15 hrs (until turbid). This culture was used to inoculate 4 flasks, each containing 4L of LB with the previously stated antibiotics at the same concentration. At an optical density of ~0.3, 200 mg of arabinose was added to each flask. At an optical density of ~0.6, the bacteria were induced with 200 μM of IPTG. Expression of the *nadA* gene was allowed to continue for 4 hrs at 37 °C. The cultures were then placed in an ice bath to cool. The cells were then harvested by centrifugation at 10,000 x g for 10 minutes. The cell paste was then frozen in liquid nitrogen and stored in a Cryo-dewar. Typical growths yielded 30-40g of frozen cell paste.

Anaerobic Purification of NadA

Cells (30g) harboring the *nadA* $C_{297}S$ variant was thawed in 80 mL of Lysis (50 mM Hepes, 0.3 M KCl, 20 mM Imidazole, 10 mM BME) inside of a Coy anaerobic chamber. The cell paste was stirred at room temperature, and once the cells were thawed, lysozyme was added to a final concentration of 1 mg mL^{-1} and the solution was allowed to stir for an additional 30 min. Cells were incubated on ice to bring the temperature to <8 °C, after which they were disrupted by sonication (4 x 1 min at a setting of 7) using a Sonicator Ultrasonic Processor (Fisher Scientific; Pittsburgh, PA). The solution was then centrifuged at 50,000-x g for 1 hour to remove cellular debris. The supernatant was loaded onto a nickel-nitrilioacetic acid (Ni-NTA) agarose column equilibrated in cold Lysis Buffer. The column was then washed with 70 mL of Wash Buffer (50 mM Hepes, 0.3 M KCl, 40 mM Imidazole, 10mM BME, 20% Glycerol). The protein was eluted from the column using Elution Buffer (50 mM Hepes, 0.3 M KCl, 250 mM Imidazole, 10mM BME, 20% Glycerol). Fractions containing protein (based on a brown color) were collected into test tubes and placed on ice. The protein was then concentrated to ~1 mL using an Amicon ultrafiltration unit with a 10,000 molecular weight membrane, and subjected to gel filtration using a G-25 column pre-equilibrated in 50 mM EPPS pH 8, 100 mM KCl, 20% glycerol, 10 mM DTT. The concentrated protein was aliquoted (200 μL) and stored in a Cryo-cooler

Protein concentration analysis

Protein concentration was determined using the method of Bradford[6] using reagent purchased from Pierce (Rockford, IL) and bovine serum albumin (BSA) as the standard. A color change of brown to blue indicated the presence of the protein.

UV-Vis spectroscopy
UV-Vis spectra were recorded using a Cary 50 (Varian; Walnut Creek, CA) in combination with the associated WinUV software package.

SDS-PAGE analysis of the protein
The purity of the protein was assessed using SDS-PAGE as described by Laemmli.[7] The molecular mass of the protein was determined using molecular mass standards obtained from Amersham (Piscataway, NJ).

Iron Analysis
The iron content in the purified NadA proteins was determined using the method of Beinert. [8] The concentration of iron was correlated to that of the protein to determine the number of irons present per polypeptide.

Results

Purification of the NadA Protein
The NadA $C_{113}S$, $C_{200}S$, $C_{291}S$, $C_{294}S$, and $C_{297}S$ variants containing a hexahistidine tag at the N-terminus were anaerobically purified. The purified proteins were brown, indicative of a Fe-S cluster. After purification, the $C_{291}S$ and $C_{294}S$ variants displayed a deep brown color, while the color of the $C_{200}S$, $C_{113}S$, and $C_{297}S$ variants was not nearly as intense. This difference suggests that cysteines 291 and 294 do not ligate the cluster whereas cysteines 200, 113, and 297 probably serve as ligands.

Spectroscopic Analysis
Spectroscopic studies were performed to determine whether the brown color of the proteins was a result of the presence of a [4Fe-4S] cluster. The UV-visible spectrum of the NaDA variants is displayed in Figure 2. As can be observed, each of the spectra shows an intense peak at 276 nm and a broader but weaker feature at 420 nm. The peak at 276 nm correlates with the protein component of each of the variants, while the feature at 420 nm correlates with the [4Fe-4S] cluster component. For the $C_{291}S$ and $C_{294}S$ variants the intensity of the peak at 420 nm was almost 25% of the peak at 276 nm, which implies that there is a substantial amount of 4Fe-4S cluster present in these proteins; however, this was not the case for the $C_{200}S$, $C_{113}S$, and $C_{297}S$ variants (ratio was less than 10%). Therefore we concluded that C_{291} and C_{294} in the wild type protein do not ligate the cluster, whereas C_{200}, C_{113}, and C_{297} may be essential for the ligation of [4Fe-4S] cluster.

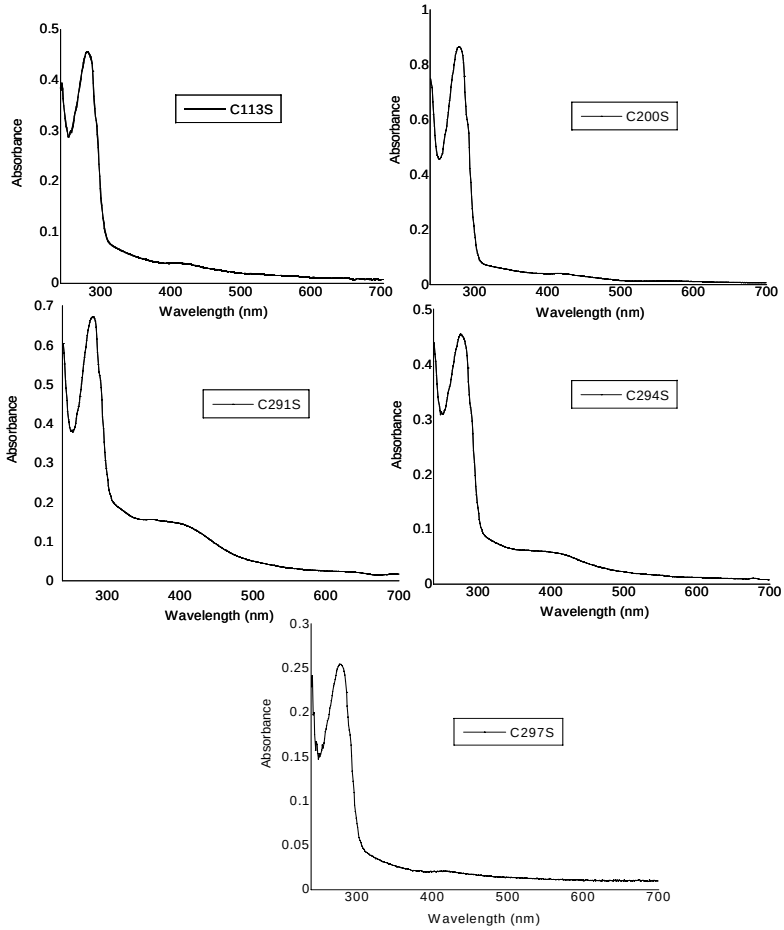

Figure 2. UV-Vis Spectra of the five variants of the NadA protein.

Further characterization of NadA was performed to determine the iron content using the method of Beinert**.** To determine the number of irons present in the protein, we compared the concentration of iron obtained to that of the protein as determined using the method of Bradford with BSA as the standard. For a [4Fe-4S] cluster it is expected that the protein should contain four irons for every polypeptide. Our results (Figure 3) indicate that the iron content was negligible for $C_{200}S$, $C_{113}S$, and $C_{297}S$ variants. We did, however, observe ~2 irons per polypeptide for the $C_{294}S$ variant and ~3 irons per polypeptide for the $C_{291}S$ variant.

Enzyme	Fe/ Protein
$C_{113}S$	< 1
$C_{200}S$	< 1
$C_{291}S$	3.27
$C_{294}S$	1.73
$C_{297}S$	< 1

Figure 3. Fe Analysis of NadA protein

Conclusion

The results of this study indicate that not all of the cysteines within the motif presumed to ligate the [4Fe-4S] cluster ($C_{291}XXC_{294}XXC_{297}$) serve as ligands, but that two of the ligands derive from other regions of the NadA protein sequence. Based on spectroscopic analysis we observed that C_{291} and C_{294} had no involvement in coordinating the cluster, whereas C_{113}, C_{200}, and C_{297} show potential as ligands to 4Fe-4S. To better confirm our preliminary findings, activity assays, sulfide analysis, electron paramagnetic resonance (EPR) spectroscopy, and Mössbauer spectroscopy will be performed on the protein.

Since the primary function of NAD is to carry out redox reactions in all living organisms its biosynthesis is crucial for cell viability. Both prokaryotes and eukaryotes biosynthesize NAD from quinolinic acid, but the pathway by which quinolinic acid produced is different between the organisms in these two kingdoms. This suggests that NadA might serve, as a potential target for the generation of antibacterial agents.[9] Thus, understanding the mechanism by which NAD is produced in bacteria is vital.

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