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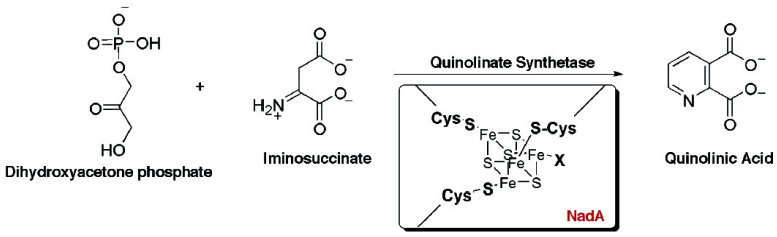
J. Am. Chem. Soc., **2005**, 127 (20), 7310-7311• DOI: 10.1021/ja051369x • Publication Date (Web): 03 May 2005



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Published on Web 05/03/2005

Escherichia coli Quinolinate Synthetase Does Indeed Harbor a [4Fe-4S] Cluster

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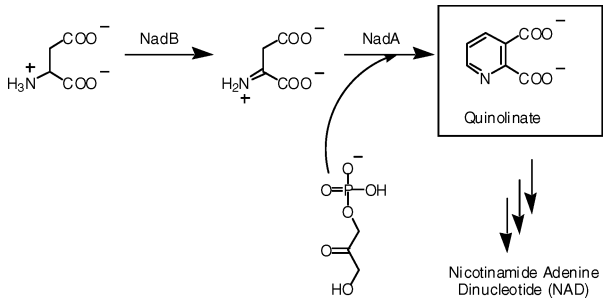
The initial steps in the biosynthesis of nicotinamide adenine dinucleotide (NAD), the primary biological cofactor for oxidation−reduction reactions, vary considerably between prokaryotes and eukaryotes, although both pathways involve the common intermediate, quinolinic acid.[1] In most eukaryotic organisms, quinolinic acid is produced via degradation of the amino acid, L-tryptophan, via an "aerobic" pathway, requiring the activities of several enzymes that use molecular oxygen as a substrate.[1,2] Alternatively, *Escherichia coli* and most other prokaryotes generate quinolinic acid via a unique condensation reaction between dihydroxyacetone phosphate (DHAP) and iminosuccinate (IS) in an "anaerobic" pathway (Scheme 1).[3,4] This reaction requires the concerted efforts of two

Scheme 1



proteins, quinolinate synthetase (NadA), and aspartate oxidase (NadB). The mechanism of NadB has been studied in fair detail; the enzyme contains a flavin cofactor, which accepts a hydride equivalent from L-aspartate, affording IS, one of the two substrates for NadA.[5−8] In contrast to NadB, there has been very little characterization of the NadA-dependent reaction or the enzyme itself, attributable in part to the sensitivity of the protein to molecular oxygen, as well as its production in inclusion bodies upon overexpression of the gene in *E. coli*,[9] characteristics that have led to the proposal that the enzyme might harbor an oxygen-sensitive iron−sulfur (Fe/S) cluster.[4] Moreover, the protein contains nine cysteine residues, three of which reside in a CX_2CX_2C motif,[10] a common pattern observed in proteins that contain [4Fe-4S] clusters, and deletion of the *E. coli iscS* gene, which is believed to mobilize sulfur for Fe/S cluster biosynthesis, renders the bacterium auxotrophic for nicotinic acid.[11] Herein, we show that coexpression of the *nadA* gene with plasmid pDB1282, which contains the *isc* operon from *Azotobacter vinelandii*, allows for production of ample amounts of soluble protein. Characterization of the protein by UV−vis, electron paramagnetic resonance (EPR), and Mössbauer spectroscopies shows unequivocally that it contains one [4Fe-4S] cluster per polypeptide.

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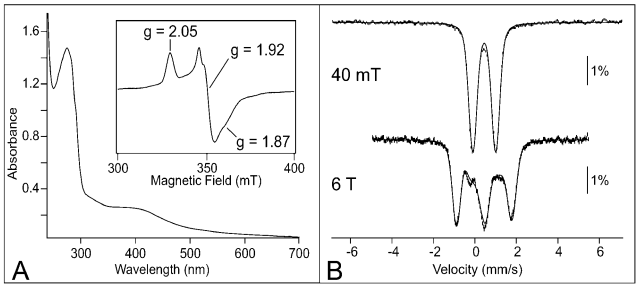


Figure 1. (A) UV−vis spectrum of anaerobically isolated NadA. Inset: EPR spectrum of as-isolated NadA, reduced in the presence of 2 mM sodium dithionite and obtained at 5 mW power and 13 K. (B) The 4.2 K Mössbauer spectra of as-isolated NadA recorded in parallel magnetic fields of 40 mT (top) and 6 T (bottom). The solid lines are simulations generated using the following parameters and assuming diamagnetism: $\delta = 0.45$ mm/s, $\Delta E_Q = 1.10$ mm/s, $\eta = 0.6$.

The *nadA* gene was cloned by PCR into plasmid pET-28A, which is IPTG-inducible, such that the protein is produced with an N-terminal hexahistidine tag. The plasmid was transformed into *E. coli* BL21(DE3), which also harbored plasmid pDB1282. The isc-related genes were induced first by addition of arabinose (0.05% final concentration) at an OD_{600} of 0.3, followed by induction of the *nadA* gene by addition of IPTG (200 μM final concentration) at an OD_{600} of 0.6. The protein was then isolated in a Coy anaerobic chamber ($\leq$1 ppm O_2) as described previously for the isolation of lipoyl synthase.[12]

NadA, overproduced and purified in this fashion, is brown in color, and its UV−vis spectrum (Figure 1A) is consistent with the presence of [4Fe-4S] clusters. To verify the presence of Fe/S clusters and accurately determine their nature and stoichiometry, anaerobically isolated NadA was characterized using a combination of Mössbauer and EPR spectroscopies and was chemically analyzed for its iron and sulfide content. The Mössbauer spectrum (Figure 1B, top spectrum, hash marks), obtained at 4.2 K and 40 mT, is a broad quadrupole doublet with parameters that are typical of a $[4Fe\text{-}4S]^{2+}$ cluster species; the isomer shift (δ) is 0.45 mm/s, and the quadrupole splitting parameter (ΔE_Q) is 1.10 mm/s (Figure 1B, top spectrum, solid line). The spectrum recorded in a 6 T parallel magnetic field (Figure 1B, bottom spectrum) can be simulated assuming a diamagnetic ($S = 0$) ground state, thereby corroborating the assignment of the quadrupole doublet to a $[4Fe\text{-}4S]^{2+}$ cluster species. The EPR spectrum of anaerobically isolated NadA (not shown) reveals the presence of a small amount (<1% of total Fe) of a $[3Fe\text{-}4S]^{+}$ cluster species, which is beyond the detection limit of Mössbauer spectroscopy. The protein contains 5.0 ± 1.4 Fe and 2.8 ± 0.3 S^{2-} per polypeptide (average and standard deviation obtained from four independent determinations). Taken together, the data suggest a stoichiometry of one [4Fe-4S] cluster per polypeptide.

10.1021/ja051369x CCC: $30.25

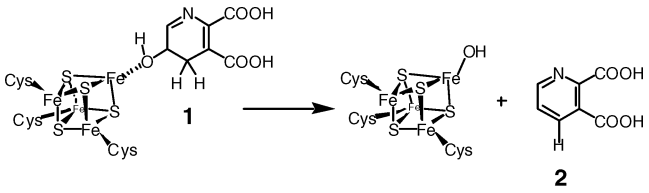
Reconstitution of NadA with additional iron and sulfide under anaerobic conditions followed by anaerobic gel-filtration chromatography results in 7.6 ± 0.9 Fe and 5.7 ± 0.5 S^{2-} per polypeptide. Analysis of the reconstituted protein by Mössbauer spectroscopy (Figure S1), however, reveals that only 40 ± 5% of the associated iron is in the form of $[4Fe\text{-}4S]^{2+}$ clusters, corresponding to a stoichiometry of 3.1 ± 0.7 irons per polypeptide in this configuration. Moreover, EPR analysis of reconstituted NadA in the absence of dithionite reduction reveals the presence of 0.1 equiv of $[4Fe\text{-}4S]^{+}$ clusters per polypeptide, corresponding to 0.4 Fe per polypeptide in this configuration. Therefore, the total stoichiometry of $[4Fe\text{-}4S]^{+,2+}$ clusters on reconstituted NadA is ~0.85 ± 0.20 per polypeptide, demonstrating that despite the presence of nine cysteines on the protein, the total number of clusters on the protein does not exceed one per polypeptide. The EPR spectrum of dithionite-reduced NadA is broad (Figure 1A, inset) and broadens further to the extent of being almost unobservable at temperatures above 50 K, consistent with its assignment as a $[4Fe\text{-}4S]^{+}$ cluster species. Spin quantification indicates that the signal accounts for 0.6 equiv of spin per polypeptide. Very little perturbation of the spectrum was observed in the presence of dihydroxyacetone phosphate or oxaloacetate, a mimic of IS, or a combination of both compounds.

The ability of both as-isolated and reconstituted NadA proteins to catalyze formation of quinolinic acid was assessed by HPLC using authentic quinolinic acid as a standard. IS, a required but unstable substrate for NadA, has a half-life of 2.5 min at 37 °C and pH 8.0[13] and, therefore, was generated from L-aspartate by action of NadB using fumarate as the electron acceptor. At pH 7.5 and 37 °C, the reconstituted enzyme catalyzed formation of quinolinic acid with a specific activity of 0.015 μmol min^{-1} mg^{-1} (V_{max} $[E_T]^{-1}$ = 0.01 s^{-1}) in an assay that was linear over 20 min (Figure S2). IS could also be generated nonenzymatically via Schiff's base formation between oxaloacetate and NH_3, added to the reaction mixture as $(NH_4)_2SO_4$;[13] however, the rate of quinolinic acid formation was 5-fold slower. To verify that quinolinic acid was indeed formed in the assay, the relevant peak from the HPLC chromatogram was collected and derivatized to allow for analysis by GC−MS. Peaks corresponding to silylated quinolinic acid and silylated nicotinic acid were observed. Nicotinic acid is a known decomposition product of quinolinic acid, which forms under the conditions of the derivatization procedure.[14] Although the apparent turnover number of the enzyme is low, it is significantly faster than the turnover number observed for the rate-limiting step in the biosynthesis of quinolinic acid via the degradation of L-tryptophan, which appears to be a nonenzymatic conversion of 2-amino-3-carboxymuconic semialdehyde to the final product via a pericyclic reaction ($k = 1.8 \times 10^{-4}$ to 2.0×10^{-3} s^{-1}).[15,16] To verify that the low activity of NadA was not due to the his-tag, activities of his-tagged NadA and non-his-tagged NadA were determined in crude lysates after normalization for expression and protein concentration. Both activities agreed with each other within a factor of two.

Our results appear to be at variance with a previously published study, in which recombinant NadA was isolated from inclusion bodies.[9] The inclusion bodies were subsequently resolubilized, purified, and refolded in the absence of added iron, affording protein that was purported to catalyze formation of quinolinic acid with a specific activity of 0.6 U mg^{-1} in the presence of EDTA, wherein one unit was defined as the amount of protein catalyzing the production of 1 mmol of NADPH per min in the assay. Given the amount of protein (60 μg) employed in the assay and the concentration of the limiting reagent (DHAP, 1 mM), we estimate that all of the substrate would have been consumed in much less than 1 min; however, the assay was conducted over a time period of 45 min. Presumably, this is simply a typographical error; however, we cannot yet draw firm comparisons between our differing activities in the absence of the true unit definition.

Two mechanisms that account for the synthesis of quinolinic acid from the condensation of DHAP and IS have been advanced, both involving the formation of species **1** (Scheme 2) as the direct

Scheme 2



precursor to quinolinic acid (species **2**).[3,8] We propose that this dehydration, which is similar to those carried out by Fe/S-containing enzymes within the hydro-lyase family,[17] is facilitated by coordination of the hydroxyl group to one of the irons of the [4Fe-4S] cluster.

The presence of distinctly different pathways in most prokaryotes and eukaryotes for the biosynthesis of quinolinic acid suggests that NadA might prove to be a key target for the design of antibacterial agents. The studies described herein show clearly that the enzyme can be obtained in sufficient amounts for both biochemical and spectroscopic characterization.

Acknowledgment. This research was supported by a grant (GM63847) from NIH to S.J.B.

Supporting Information Available: Experimental procedures, the Mössbauer spectrum of reconstituted NadA, and a graph showing the time-dependent formation of quinolinic acid. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Magni, G.; Amici, A.; Emanuelli, M.; Rafaelli, N.; Ruggieri, S. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1999**, *73*, 135−182.
(2) Kurnasov, O.; Goral, V.; Colabroy, K. L.; Gerdes, S.; Anantha, S.; Osterman, A.; Begley, T. P. *Chem. Biol.* **2003**, *10*, 1195−1204.
(3) Begley, T. P.; Kinsland, C.; Mehl, R. A.; Osterman, A.; Dorrestein, P. *Vitam. Horm.* **2001**, *61*, 103−119.
(4) Penfound, T.; Foster, J. W. In *Escherichia coli and Salmonella*; Neidhardt, F. C., Ed.; American Society for Microbiology: Washington, DC, 1996; Vol. 1, pp 721−730.
(5) Tedeschi, G.; Rafaelli, N.; Simonic, T.; Treu, C.; Mattevi, A.; Negri, A. *Biochemistry* **2001**, *40*, 4738−4744.
(6) Bossi, R. T.; Negri, A.; Tedeschi, G.; Mattevi, A. *Biochemistry* **2002**, *41*, 3018−3024.
(7) Mortarino, M.; Negri, A.; Tedeschi, G.; Simonic, T.; Duga, S.; Gassen, H. G.; Ronchi, S. *Eur. J. Biochem.* **1996**, *239*, 418−426.
(8) Nasu, S.; Wicks, F. D.; Gholson, R. K. *J. Biol. Chem.* **1982**, *257*, 626−632.
(9) Ceciliani, F.; Camori, T.; Ronchi, S.; Tedeschi, G.; Mortarino, M.; Galizzi, A. *Prot. Express. Purif.* **2000**, *18*, 6−70.
(10) Flachmann, R.; Kunz, N.; Seifert, J.; Gutlich, M.; Wientjes, F. J.; Laufer, A.; Gassen, G. *Eur. J. Biochem.* **1990**, *175*, 221−228.
(11) Lauhon, C. T.; Kambampati, R. *J. Biol. Chem.* **2000**, *275*, 20096−20103.
(12) Cicchillo, R. M.; Iwig, D. F.; Jones, A. D.; Nesbitt, N. M.; Baleanu-Gogonea, C.; Souder, M. G.; Tu, L.; Booker, S. J. *Biochemistry* **2004**, *43*, 6378−6386.
(13) Nasu, S.; Gholson, R. K. *Biochem. Biophys. Res. Commun.* **1981**, *101*, 533−539.
(14) McDaniel, H. G. *Methods Enzymol.* **1980**, *66*, 91−95.
(15) Keys, I. L. D.; Hamilton, G. A. *J. Am. Chem. Soc.* **1987**, *109*, 2156−2163.
(16) Colabroy, K. L.; Begley, T. P. *J. Am. Chem. Soc.* **2005**, *127*, 840−841.
(17) Flint, D. H.; Allen, R. M. *Chem. Rev.* **1996**, *96*, 2315−2334.

JA051369X