VIP NAD Biosynthesis

DOI: 10.1002/anie.201202261

Studies of Inhibitor Binding to the [4Fe-4S] Cluster of Quinolinate Synthase**

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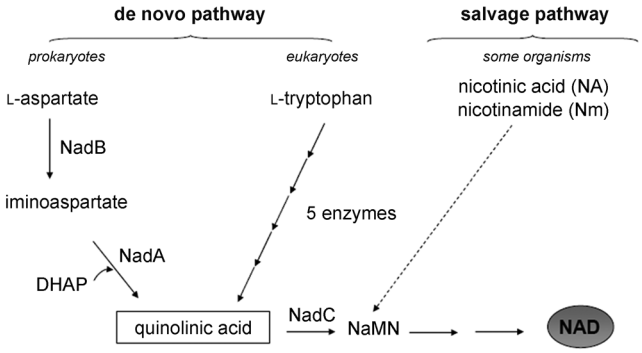
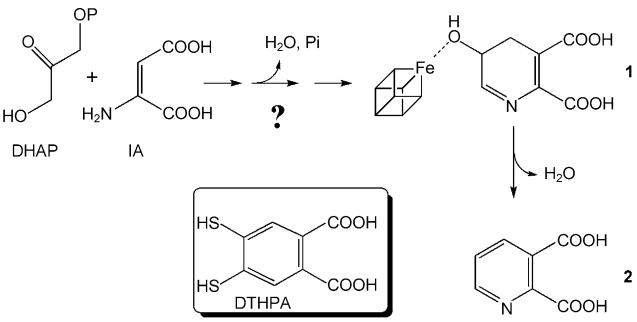
Nicotinamide adenine dinucleotide (NAD) is an essential and ubiquitous cofactor known for its role as a cosubstrate in biological oxidation–reduction reactions that involve hydride transfer.[1] It is also involved in a myriad of non-redox reactions such as adenylation, ADP ribosylation, and histone deacetylation.[2,3] NAD biosynthesis involves the formation of quinolinic acid (QA; Scheme 1), the biosynthesis of which is different in prokaryotes and eukaryotes. In most eukaryotic organisms, QA is produced by the oxidative degradation of L-tryptophan, which is mediated by five enzymes.[4] In most prokaryotes, including *Escherichia coli*, and plants QA is generated by a unique condensation reaction between dihydroxyacetone phosphate (DHAP) and iminoaspartate (IA). This reaction requires the concerted actions of two proteins, L-aspartate oxidase (NadB), which first converts L-aspartate into iminoaspartate, and quinolinate synthase (NadA), which condenses IA with DHAP to form QA. In the latter process, water (2 mol) and inorganic phosphate (1 mol) are produced (Scheme 2).[5] Besides these de novo syntheses of NAD, a salvage pathway exists in some organisms that enables NAD to be recycled from nicotinic acid (NA) and nicotinamide (Nm).[6] However, some pathogens, such as *Mycobacterium leprae*, which is the causative agent of leprosy, and *Helicobacter pylori*, which is a major cause of gastroduodenal diseases, were reported to lack this salvage pathway.[7] The presence in most prokaryotes and eukaryotes of distinct pathways for quinolinic acid biosynthesis, along with the absence of the salvage pathway in some pathogenic microorganisms, makes NadA a novel target for the development of specific antibacterial drugs.



Scheme 1. Summary of the pathways leading to the formation of NAD in living organisms. DHAP = dihydroxyacetone phosphate, NAD = nicotinamide adenine dinucleotide, NadA = quinolinate synthase, NadB = L-aspartate oxidase, NadC = quinolinic acid phosphoribosyltransferase, NaMN = mononucleotide nicotinic acid.



Scheme 2. Proposed role of the NadA [4Fe-4S] cluster in the last step of the mechanism of formation of QA from DHAP and IA. Inset shows 4,5-dithiohydroxyphthalic acid (DTHPA). IA = iminoaspartate, Pi = inorganic phosphate.

NadA from *E. coli* has served as the prototype for the study of quinolinate synthases. We and others have shown that the isolated protein binds one oxygen-sensitive $[4Fe\text{-}4S]^{2+}$ cluster that is essential for activity,[8,9] and which is bound by only three strictly conserved cysteines.[10] This situation is reminiscent of those found in dehydratases, such as aconitase, or in Radical-SAM enzymes. In these systems, the fourth coordination site is occupied by the cognate substrate: in the case of aconitase, this is citrate bound through its hydroxyl and carboxylate groups, and in the case of Radical-SAM enzymes, this is the amino and carboxylate groups of the SAM methionine moiety.[11,12] This suggests that one iron site of the NadA [4Fe-4S] cluster could similarly

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[**] J.M.L. acknowledges the support of the Région Rhône-Alpes through contract CIBLE 07 016335.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201202261.

interact with substrates or intermediates during QA formation.

Two mechanisms that account for the synthesis of QA from the condensation of DHAP and IA have been proposed in the literature, differing in the way DHAP is condensed with IA. However, both mechanisms involve the formation of **1** (Scheme 2), which is bound to the [4Fe-4S] cluster. Dehydration of **1** would form quinolinic acid (**2**, Scheme 2), but no experimental support has been provided for any of them to date.[6,13] This dehydration, which is similar to those carried out by Fe/S-containing enzymes within the hydrolyase family, would be facilitated by coordination of the hydroxyl group of **1** to the non-cysteinyl iron of the [4Fe-4S] cluster, which acts as a Lewis acid (Scheme 2).

Although it is often suggested, the existence of a differentiated Fe site in the NadA [4Fe-4S] cluster, which is involved in the catalysis of QA formation, has never been demonstrated. To address this point, we synthesized 4,5-dithiohydroxyphthalic acid (DTHPA), a structural analogue of **1** (inset, Scheme 2). A combination of in vitro and in vivo inhibition studies, as well as Mössbauer experiments supported by DFT calculations revealed that DTHPA coordinates to the NadA [4Fe-4S] cluster through a differentiated iron site, thus leading to the inhibition of QA formation. Herein, we report the results and insights gained from this study.

Under anaerobic conditions, we measured the in vitro activity of *E. coli* NadA in the presence of DTHPA by HPLC quantification of QA formation. We observed a loss of NadA activity as a function of DTHPA concentration (Figure 1).

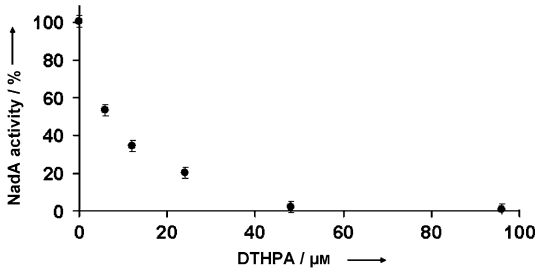

Figure 1. Enzymatic activity of NadA (7 μM; from *E. coli*) in the presence of DTHPA (0-96 μM). Each point is the average of three experiments.

From these data an IC_{50} value of 7 μM was obtained, which corresponds to a 1:1 ratio with regards to protein concentration, and reveals the strong inhibitory power of DTHPA towards NadA. This is the first reported example of an in vitro inhibitor of NadA.

To investigate DTHPA inhibition at a molecular level, we performed spectroscopic analyses on Fe/S-containing NadA in the presence of DTHPA. Upon addition of 6 equiv of DTHPA, the UV/Vis spectrum of NadA is modified (Figure 2a). The prominent $S \rightarrow Fe^{3+}$ charge transfer band at 420 nm, which is characteristic of a NadA $[4Fe\text{-}4S]^{2+}$ cluster, is less defined and slightly blue-shifted compared to that obtained in the absence of DTHPA. Furthermore, a small

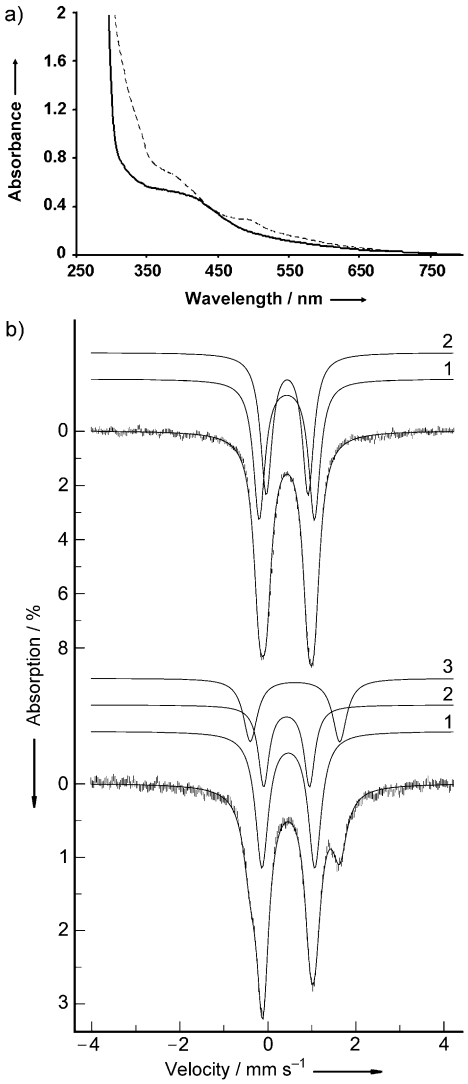

Figure 2. a) UV/Vis spectrum of ^{57}Fe/S–NadA (300 μM) in Tris-HCl (100 mM, pH 7.5), NaCl (50 mM) without (——) or with (-----) DTHPA (1.8 mM). b) Mössbauer spectra of ^{57}Fe/S–NadA in the absence (top) and in the presence (bottom) of DTHPA (6 equiv). Taken at 4.2 K with an external magnetic field of 0.06 T applied parallel to γ rays. Traces 1, 2, and 3 represent component contributions (see text for details).

absorption band around 500 nm appears in the presence of DTHPA. The change in the UV/Vis spectrum indicates that DTHPA interacts with the NadA $[4Fe\text{-}4S]^{2+}$ cluster. This was confirmed by Mössbauer spectroscopy. Figure 2b (top) displays a Mössbauer spectrum of isolated NadA recorded at 4.2 K with a small (0.06 T) magnetic field applied parallel to the γ rays. It consists of a quadrupole doublet that can be fitted with two components of equal intensity (component 1: $\delta = 0.44\ mm\,s^{-1}$, $\Delta E_Q = 1.25\ mm\,s^{-1}$; component 2: $\delta = 0.45\ mm\,s^{-1}$, $\Delta E_Q = 0.95\ mm\,s^{-1}$). These parameters are characteristic of delocalized mixed valence $Fe^{2.5+}$ pairs within diamagnetic $[4Fe\text{-}4S]^{2+}$ clusters. Accordingly, a high field experiment confirmed the spin $S = 0$ of this species (Supporting Information, Figure S1 and Table S1). Note that the non-cysteine-bound Fe of the cluster is not distinguished in the Mössbauer spectrum, as has sometimes been previously

observed.[14] The addition of 6 equiv of DTHPA to Fe/S-containing NadA causes drastic changes in the Mössbauer spectrum, as shown in Figure 2b (bottom). Indeed, the low field spectrum shows that the central quadrupole doublet is now flanked by a shoulder at low velocity and presents an additional peak at high velocity. Both features reveal the presence of an additional component with larger quadrupole splitting. The spectrum could be fitted adequately by taking into account three components with an intensity ratio of 2:1:1. Component 1 possesses parameters associated with a delocalized pair ($\delta = 0.48$ mm s^{-1}, $\Delta E_Q = 1.20$ mm s^{-1}). Component 2 ($\delta = 0.44$ mm s^{-1}, $\Delta E_Q = 1.04$ mm s^{-1}) and component 3 ($\delta = 0.63$ mm s^{-1}, $\Delta E_Q = 2.02$ mm s^{-1}) constitute a pair of Fe atoms that are partially localized, as indicated by the more ferrous character of component 3 in particular.[15] Again the high field Mössbauer spectrum (Figure S2 and Table S1) confirmed the diamagnetic ground state of the species expected for a $[4Fe\text{-}4S]^{2+}$ cluster. The spectrum of Figure 2b (bottom) is highly reminiscent of that observed for $[4Fe\text{-}4S]^{2+}$ clusters having a differentiated iron site, as found in aconitase and a few other enzymes,[16–20] which strongly suggests that the NadA cluster also possesses a differentiated Fe site that interacts with potent ligands. The Mössbauer parameters of component 3 ($\delta = 0.63$ mm s^{-1}, $\Delta E_Q = 2.02$ mm s^{-1}) are nearly identical to those reported by Ciurli et al.[21] for a $[4Fe\text{-}4S]^{2+}$ chemical model containing a differentiated iron site bound by 4-toluene-1,2-dithiolate through the two sulfur atoms ($\delta = 0.63$ mm s^{-1}, $\Delta E_Q = 1.97$ mm s^{-1}).[22] Therefore, our Mössbauer analysis shows that DTHPA interacts with a differentiated iron site of the NadA $[4Fe\text{-}4S]^{2+}$ cluster, and strongly suggests that this interaction occurs through the two thiolate groups of DTHPA.

To further validate our conclusions we performed DFT calculations on the Mössbauer parameters of a model (Figure 3) consisting of DTHPA bound to a [4Fe-4S] cluster through a differentiated Fe site, while the other three iron atoms are bound to three ethyl thiolates, which mimic the three cysteine residues (see the Supporting Information for details). The quadrupole splittings computed for this DTHPA-bound model with protonated carboxylate groups reproduced the ones measured experimentally for NadA, in particular for the unique iron site (2.01–2.21 mm s^{-1} vs. an experimental value of 2.02 mm s^{-1}; Table S2). This lends further credence to the chelated binding mode of the dithiolate moiety shown in Figure 3.

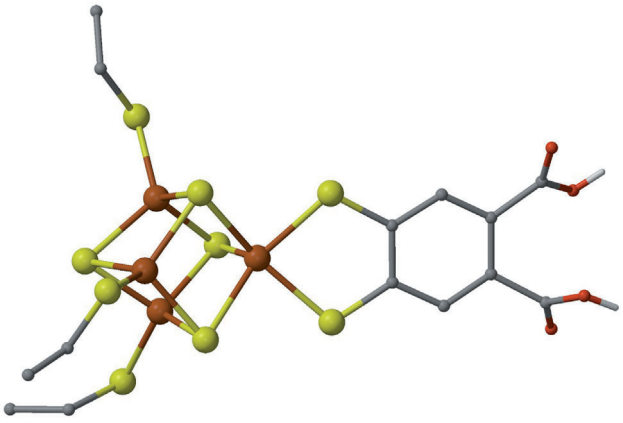

Figure 3. Model for a DTHPA-bound NadA [4Fe-4S] cluster with protonated carboxylate groups. Fe atoms in brown, O atoms in red, S atoms in yellow.

Finally, we investigated if DTHPA could be an in vivo inhibitor. First, we established that in a minimal medium devoid of NA and Nm, a Δ*nadA E. coli* strain that is unable to grow can be rescued by the addition of QA (Figure S6). This strain mimics the situation of a wild-type *E. coli* strain in which the NadA protein would be inhibited. Then, we monitored the wild-type *E. coli* strain growth as a function of DTHPA concentration in a minimal medium (Figure 4). The OD_{600nm} values show that the bacterial growth is affected from 10 μM DTHPA and totally inhibited with 20 μM DTHPA, a concentration defining the minimal inhibitory concentration. Moreover, whereas bacterial growth can be rescued by the addition of QA when using DTHPA in the range of 10–20 μM, it is not the case beyond 20 μM DTHPA, which suggests that at low concentrations DTHPA is a selective in vivo inhibitor of QA formation (Figure 4). At higher concentrations, DTHPA likely inhibits other essential metalloproteins, thus leading to bacterial death even in the presence of QA.

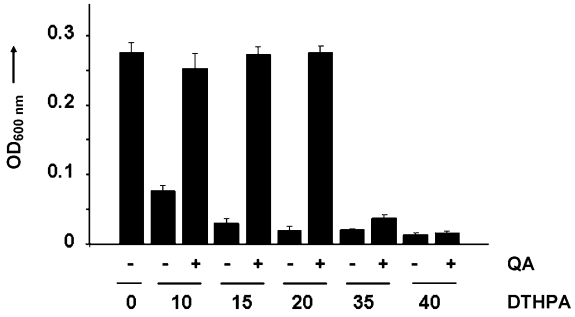

Figure 4. Selective inhibition of QA formation in vivo by DTHPA. OD_{600nm} of *E. coli* MG1655 after 16 h of growth in a minimal medium in the presence of increasing amounts of DTHPA (μM). Each value is the average of five experiments.

In conclusion, we report a study of the $[4Fe\text{-}4S]^{2+}$ cluster of NadA in the presence of 4,5-dithiohydroxyphthalic acid, an analogue of a postulated intermediate in QA production. Using both in vitro Mössbauer spectroscopy and DFT calculations we demonstrate that DTHPA binds to the NadA cluster through its thiolate moiety, thus bringing to light, for the first time, the existence of a differentiated iron site. The fact that DTHPA binding is associated with the loss of NadA activity in vitro demonstrates that the chemistry of QA formation, the DHAP and IA condensation, occurs at the $[4Fe\text{-}4S]^{2+}$ cluster through its differentiated iron. Identification of the fourth ligand of the cluster in the resting state is underway in our laboratory, as well as investigation of DTHPA acting as an inhibitor of NadA proteins from pathogens such as *M. leprae* and *H. pylori*. Indeed, our in vivo data on the *E. coli* strain raises the possibility of

DTHPA acting as a new antibacterial agent on a new target quinolinate synthase.

Received: March 22, 2012
Published online: June 19, 2012

Keywords: inhibitors · iron-sulfur clusters · Moessbauer spectroscopy · NAD · quinolinate

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