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Structure of Quinolinate Synthase from *Pyrococcus horikoshii* in the Presence of Its Product, Quinolinic Acid

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Supporting Information

ABSTRACT: Quinolinic acid (QA) is a common intermediate in the biosynthesis of nicotinamide adenine dinucleotide (NAD^+) and its derivatives in all organisms that synthesize the molecule *de novo*. In most prokaryotes, it is formed from the condensation of dihydroxyacetone phosphate (DHAP) and aspartate-enamine by the action of quinolinate synthase (NadA). NadA contains a [4Fe-4S] cluster cofactor with a unique, non-cysteinyl-ligated, iron ion (Fe_a), which is proposed to bind the hydroxyl group of a postulated intermediate in the last step of the reaction to facilitate a dehydration. However, direct evidence for this role in catalysis has yet to be provided. Herein, we present the structure of NadA in the presence of the product of its reaction, QA. We find that N1 and the C7 carboxylate group of QA ligate to Fe_a in a bidentate fashion, which is confirmed by Hyperfine Sublevel Correlation (HYSCORE) spectroscopy. This binding mode would place the C5 hydroxyl group of the postulated final intermediate distal to Fe_a and virtually incapable of coordinating to it. The structure shows that three strictly conserved amino acids, Glu198, Tyr109, and Tyr23, are in close proximity to the bound product. Substitution of these amino acids with Gln, Phe, and Phe, respectively, leads to complete loss of activity.

Quinolinate synthase (NadA) catalyzes the condensation of dihydroxyacetone phosphate (DHAP) and aspartate-2,3-ene (aspartate-enamine; Asp-EA) to afford quinolinic acid (QA), orthophosphate, and 2 molecules of water (Figure 1). QA is a common precursor in the biosynthesis of NAD^+ among all organisms that synthesize the molecule and its derivatives *de novo*. However, with some exceptions, the biosynthetic pathway for formation of QA differs in eukaryotes and prokaryotes.[1] In the prokaryotic pathway, often termed the anaerobic pathway, NadA acts in concert with L-aspartate (Asp) oxidase (NadB), a flavin-dependent oxidase/dehydrogenase that catalyzes the two-electron oxidation of Asp to Asp-EA with concomitant transfer of two electrons to flavin adenine dinucleotide (FAD) to afford the reduced flavin, $FADH_2$.[2−5] Under aerobic conditions, molecular oxygen is used to oxidize $FADH_2$ back to FAD, while under anaerobic conditions, $FADH_2$ is oxidized by fumarate, resulting in the production of succinate.[6−8] In the eukaryotic pathway, L-tryptophan is converted into QA in a series of steps that require molecular oxygen.[1]

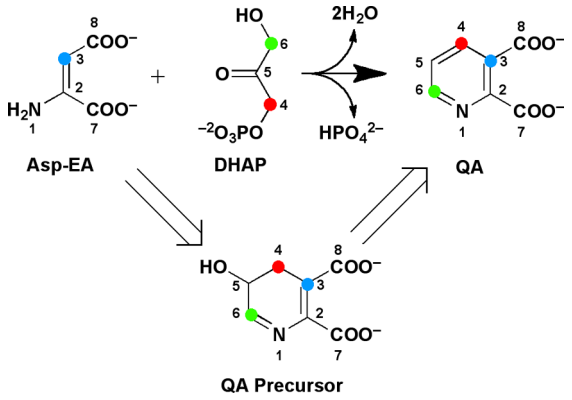

Figure 1. NadA-catalyzed condensation of Asp-EA and DHAP to give QA. The colored dots indicate known connectivity between atoms. QA is numbered according to IUPAC rules, and this numbering is maintained in the DHAP and Asp-EA structures for clarity.

The mechanism by which NadA catalyzes its reaction has been elusive since the discovery of its activity in the late 1960s and early 1970s.[2,5,9] Studies by Gholson and colleagues indicate a connectivity of C3 of Asp-EA (shown as C3 in Figure 1) to C1 of DHAP (shown as C4 in Figure 1), and the nitrogen of Asp-EA to C3 of DHAP (shown as C6 in Figure 1).[10] NadA contains one [4Fe-4S] cluster cofactor per monomer, in which each of three of the iron ions of the cluster is ligated by a cysteinyl residue.[11−15] It belongs to the iron−sulfur (Fe/S)-dependent hydro-lyase family of enzymes, of which aconitase is the paradigm.[16,17] Enzymes in this family are all believed to use the unique, noncysteinyl-ligated, iron ion (Fe_a) as a Lewis acid to facilitate hydration/dehydration reactions.[17]

Two mechanistic hypotheses for catalysis by NadA have been advanced (Figure S1).[1,4] In the earlier of the two, proposed by Gholson and colleagues, C3 of the enamine of Asp-EA attacks C4 of DHAP, resulting in the elimination of orthophosphate and the formation of the nascent C−C bond of QA. Subsequent tautomerizations and Schiff-base formation between the amine of the formerly Asp-EA and the C6 aldehyde of the formerly DHAP lead to production of the QA precursor (QP).[4] The C5 hydroxyl of the QP is proposed to ligate to Fe_a to facilitate a dehydration reaction that is initiated by proton removal at C4.[17]

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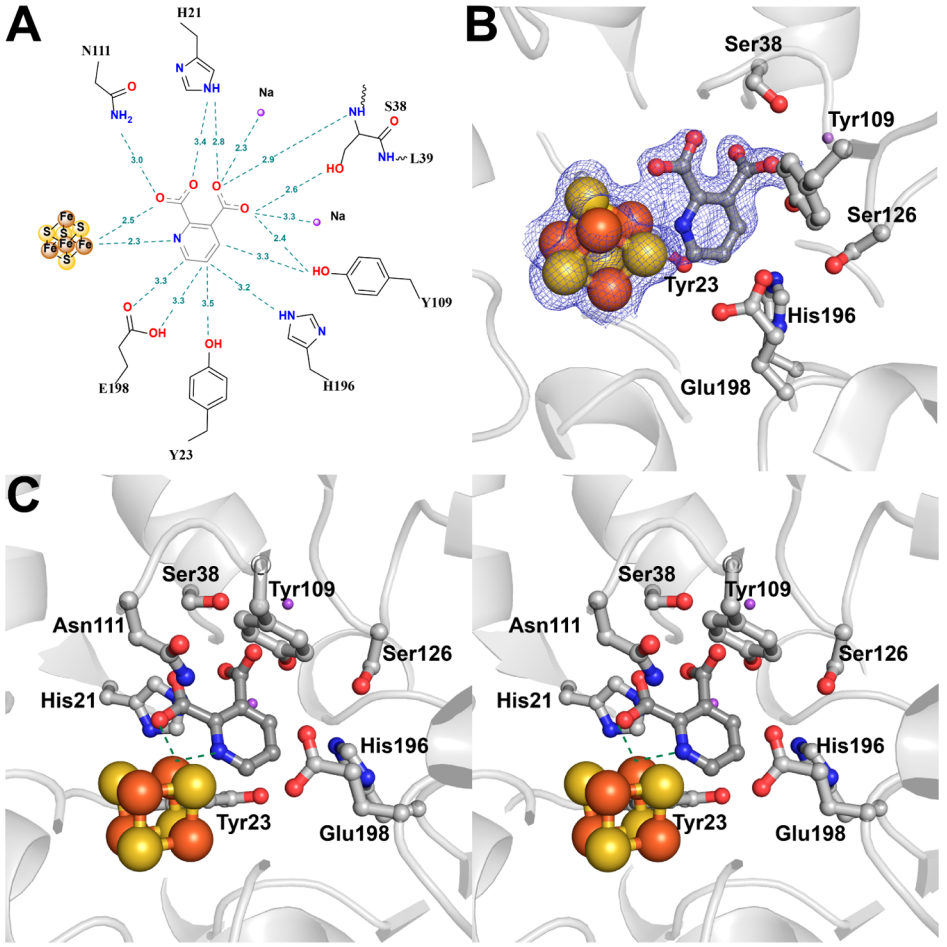

Figure 2. (A) Schematic diagram of QA in the active site of *Ph*NadA, showing that the N1 nitrogen and C8 carboxylate ligate to Fe_a in a bidentate fashion, forming a five-membered chelate. (B) Omit electron density map of QA in the active site of *Ph*NadA. The $2F_o - F_c$ electron density is contoured at 1.0σ (blue mesh). (C) Cross-eyed stereoimage showing the interactions of QA with the Fe/S cluster and the placement of conserved amino acids in the active site of *Ph*NadA. Atom colors: Fe, orange; S, yellow; N, blue; C, gray; O, red; and Na, magenta.

In a second mechanism, suggested by Begley and colleagues (Figure S2), the first step is generation of glyceraldehyde 3-phosphate (G3P) through tautomerizations of DHAP, which is followed by Schiff-base formation between Asp-EA and the aldehyde of G3P. Loss of phosphate, via an elimination reaction, sets up a pericyclic system that results in formation of the nascent C−C bond in QA. Tautomerization of the resulting species affords the QP, which is proposed to undergo dehydration with the assistance of Fe_a, as described above.[1]

Interestingly, all enzymes within the Fe/S-dependent hydrolyase family operate on substrates that contain carboxylate groups.[17] In the structure of the S642A variant of bovine mitochondrial aconitase in complex with citrate, both the C3 hydroxyl and the C3 carboxylate of citrate bind to Fe_a, forming a five-membered ring and giving rise to an octahedral geometry about the metal ion, which is also coordinated by a water or hydroxide ligand.[18] By contrast, the hydroxyl group at C5 of the proposed QP should be geometrically constrained from binding to Fe_a simultaneously with either of the carboxylate groups, raising the question as to which of these two interactions is mechanistically important.

In an effort to provide insight into the role of its Fe/S cluster in catalysis, we set out to solve the X-ray structure of wild-type NadA from *Pyrococcus horikoshii* (*Ph*) in the presence of QA (Table S1). Crystals of *Ph*NadA in complex with QA were obtained by including QA (10 mM) both in the screening solution and in the solution for cryoprotection (Table S2). The structure, which is dimeric in the asymmetric unit, was solved to a resolution of 1.9 Å by molecular replacement using the coordinates of the apo *Ph*NadA structure (PDB 1WZU) as the search model.[20] The overall three-dimensional architecture of the *Ph*NadA/QA structure is almost identical to the structure of *Ph*NadA lacking its Fe/S cluster but containing a molecule of bound malate. The structure consists of a single polypeptide that displays pseudo-three-fold symmetry, as shown previously in the structures of apo *Ph*NadA (RMSD of 1.288 Å) and NadA from *Thermotoga maritima* (*Tm*NadA) (RMSD of 5.740 Å) (Table S3).[15,19] In the *Ph*NadA/QA structure, the Fe/S cluster is at the center of the three domains, and is ligated by cysteines 83, 170, and 256. These cysteines lie on loops that connect each of the domains, as shown in previous biochemical studies as well as in the structure of *Tm*NadA.[12,14,15]

In the active site of the structure, electron density that is consistent with QA was observed (Figure 2B); however, the orientation of the molecule was unclear due to its C2 axis of symmetry, given the inability to distinguish a nitrogen atom from a carbon atom. To address this ambiguity, we employed Hyperfine Sublevel Correlation (HYSCORE) spectroscopy to ascertain whether the nitrogen atom of QA is coordinated to the Fe/S cluster. A solution of *Ph*NadA containing 20 mM QA

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was incubated with dithionite for 3 min to promote the reduction of the Fe/S cluster to the EPR-active +1 oxidation state before loading it into an EPR tube and freezing it rapidly in cryogenic isopentane. X-band HYSCORE spectra of the *Ph*NadA/QA sample contain signals that are clearly evident of an ^{14}N nucleus at a bonding distance to the $[4Fe\text{-}4S]^{+}$ cluster (Figure 3B). Moreover, these signals are absent in a control

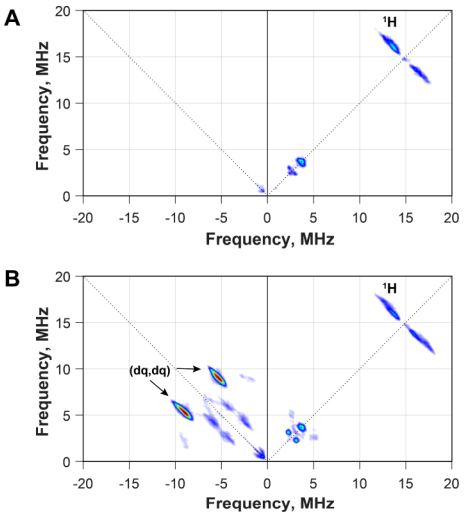

Figure 3. X-band HYSCORE spectra of dithionite reduced NadA in absence (A) and presence (B) of quinolinic acid, taken at the maximum absorption of the EPR spectrum of the $[4Fe\text{-}4S]^{+}$ cluster (g = 1.94). Experimental conditions: temperature, 10 K; microwave frequency, 9.426 GHz; magnetic field, 347 mT; delay between first two pulses (τ), 138 ns; $t(\pi/2)$, 8 ns; shot repetition time, 0.5 ms.

sample that was prepared in the absence of QA (Figure 3A). The spectrum is dominated by double quantum correlation ridges (dq,dq) that are centered at −5.5, +9.1 MHz and −9.1, +5.5 MHz in the (−/+) quadrant of the HYSCORE spectrum, indicating a strong coupling case, given that the hyperfine (HF) coupling constant is larger than twice the Larmor frequency. Using these frequency values ($\nu_{dq,\pm}$), the isotropic component of the ^{14}N HF coupling constant (a_N) was estimated using the relations shown in eqs 1 and 2, where ν_N is the Larmor

$$\nu_{dq,\pm} = 2\left(\left(\nu_N \pm \frac{|a_N|}{2}\right)^2 + K^2(3+\eta^2)\right)^{1/2} \quad (1)$$

$$|a_N| = \left|\frac{\nu_{dq,+}^2 - \nu_{dq,-}^2}{8\nu_N}\right| = 6.1 \text{ MHz} \quad (2)$$

frequency of the ^{14}N nucleus (1.07 MHz at 347 mT), and K and η are the magnitude and rhombicity, respectively, of the quadrupole coupling.[20] This value is very close to the values found for the bidentate binding of SAM to the [4Fe-4S] cluster in radical SAM (RS) enzymes such as lysine 2,3-aminomutase (a_N = 6.5 MHz; recalculated from ^{15}N coupling constants)[21] and TYW1 (a_N = 5.6 MHz).[22]

In the structure of *Ph*NadA/QA, N1 and the C7 carboxylate of QA coordinate to Fe_a of the Fe/S cluster—as unambiguously established by HYSCORE spectroscopy—while C5, originally proposed to bear the hydroxyl group of the QP, is distal to Fe_a, on the opposite side of the C2 axis of symmetry. The C8 carboxylate forms H-bonds to the side chains of Tyr109, His21, and Ser38, as well as to the backbone amide of Ser38 (Figure 2A). It also forms electrostatic interactions with two sodium ions. Several strictly conserved residues have side chains that are in close proximity to QA and that likely perform key roles in the last few steps of the reaction. Tyr109 lies 3.3 Å above the *si* face of C3 of QA and is also 3.3 Å away from C4, suggesting that it may be involved in formation of the C−C bond between Asp-EA and DHAP. Tyr23 lies below the opposite face of QA 3.8, 3.5, and 4.0 Å away from C6, C5, and C4, respectively, while Glu198 lies 3.3 Å both above C6 and above C5. Activity determinations show that all three of these amino acids are essential in catalysis, given that the Tyr23Phe, Tyr109Phe, and Glu198Gln variants are inactive within the limits of detection of our assay (Table S4). An overlay of the *Ph*NadA/QA and apo *Ph*NadA/malate structures (Figure S3) shows that the absence of the Fe/S cluster in the latter structure causes the C2−C3 bond of malate to rotate ∼150° to form H-bonds with conserved residues Tyr109, Ser28, and Ser126.

The role of the Fe/S cluster as a Lewis acid in the NadA reaction has been presumed based on studies of aconitase, but has never been established unambiguously. However, until now, there have been no structures of enzymes in this family with bound ligands—except for aconitase—to investigate the role of the Fe/S cluster more widely. Perplexingly, all enzymes within this family operate only on substrates that contain carboxyl groups, although there is no inherent chemical rationale for this substrate preference. In aconitase, which catalyzes a reversible dehydration and subsequent rehydration following a major rearrangement of an intermediate in the active site, both the hydroxyl group that is lost and a carboxylate group of the substrate coordinate to Fe_a simultaneously, forming a five-membered bis-chelate. However, in a number of aconitase family members such as L-serine deaminase, dihydroxy-acid dehydratase, phosphogluconate dehydratase, and NadA, simultaneous coordination cannot take place or would not be consistent with the appropriate transition state for an E1cb elimination reaction.[17] Although other enzymes in this superfamily have yet to be studied in detail, our studies show that, at least in the NadA reaction, carboxylate coordination by the substrate is maintained, while hydroxyl coordination is unlikely in the expected last step of the reaction.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b02708.

Tables S1−S5 and Figures S1−S3 (PDF)

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Notes

The authors declare no competing financial interest.

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