Regulation of the Activity of *Escherichia coli* Quinolinate Synthase by Reversible Disulfide-Bond Formation†

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ABSTRACT: Quinolinate synthase (NadA) catalyzes a unique condensation reaction between dihydroxyacetone phosphate and iminoaspartate, yielding inorganic phosphate, 2 mol of water, and quinolinic acid, a central intermediate in the biosynthesis of nicotinamide adenine dinucleotide and its derivatives. The enzyme from *Escherichia coli* contains a $C^{291}XXC^{294}XXC^{297}$ motif in its primary structure. Bioinformatics analysis indicates that only Cys297 serves as a ligand to a [4Fe-4S] cluster that is required for turnover. In this report, we show that the two remaining cysteines, Cys291 and Cys294, undergo reversible disulfide-bond formation, which regulates the activity of the enzyme. This mode of redox regulation of NadA appears physiologically relevant, since disulfide-bond formation and reduction are effected by oxidized and reduced forms of *E. coli* thioredoxin. A midpoint potential of -264 ± 1.77 mV is approximated for the redox couple.

Quinolinic acid (QA) is a key intermediate in the biosynthesis of nicotinamide adenine dinucleotide (NAD^+) and its derivatives in all organisms that synthesize the cofactor de novo. The mechanism by which this common precursor is biosynthesized, however, differs between most, but not all, eukaryotes and prokaryotes (*1*). Vertebrates, and most other eukaryotes, synthesize QA via the degradation of L-tryptophan. By contrast, prokaryotes and a small number of eukaryotes synthesize QA via a unique condensation reaction between dihydroxyacetone phosphate (DHAP) and iminoaspartate (IA), which is catalyzed by quinolinate synthase (NadA) (*1*). IA is generated by the action of L-aspartate oxidase (NadB), an enzyme that requires flavin adenine dinucleotide (FAD). During turnover, FAD is transiently reduced by two electrons to $FADH_2$ (*2*). Under aerobic conditions, the flavin is reoxidized by molecular oxygen, affording production of hydrogen peroxide. Under anaerobic conditions, fumarate acts as the oxidant and is converted to succinate (Scheme S1) (*3*).

IA is labile and has been reported to be hydrolyzed to oxaloacetate (OAA) and ammonia with a half-life of 140 s (*2*). Studies by Nasu and Gholson have shown that the NadB protein can be replaced by addition of OAA and an ammonia source, such as ammonium sulfate, which gives rise to an equilibrium concentration of IA via Schiff base formation (*4*).

In early mechanistic studies of NadA from *Escherichia coli*, the enzyme was reported to be unstable, especially in the presence of hyperbaric oxygen (*5, 6*). This characteristic, as well as a CXXCXXC motif found in its primary structure, led to the suggestion that it might contain an iron−sulfur (Fe−S) cluster (*6*). Indeed, recent characterization of the protein purified under anaerobic conditions showed that it harbors a [4Fe-4S] cluster that is absolutely required for activity (*7, 8*). Bioinformatics analysis suggests, however, that only one of the Cys residues in the $C^{291}XXC^{294}XXC^{297}$ motif (Cys297) is a ligand to the cluster (*9*). The remaining two cysteines, Cys291 and Cys294, exist in a motif that is commonly found in proteins that undergo reversible disulfide-bond formation. In recent studies of *E. coli* NadA (*7, 8*), the protein was kept under anoxic and reducing conditions because of its reported lability in the presence of oxygen (*6*). This observed oxygen sensitivity was later reproduced; exposure of the protein to oxygen resulted in loss of the [4Fe-4S] cluster and its characteristic UV−vis signal, as well as loss of the enzyme's ability to catalyze the reaction (*8*). Given that NadB uses dioxygen as a cosubstrate under aerobic conditions, catalyzing the formation of peroxide, another reactive oxygen species (ROS) that is known to have deleterious effects on Fe−S clusters (*10*), we reinvestigated the oxygen sensitivity of NadA and found that the activity of the protein is actually greater under oxic conditions and is regulated by a redox-active disulfide bond.

In Figure S1 (see the Supporting Information), a NadA-dependent activity determination under oxic and anoxic conditions is displayed, in which L-aspartate and the NadB protein are replaced by addition of 100 mM ammonium chloride and 5 mM OAA, which affords a saturating concentration of IA. The use of chemically generated IA in place of NadB obviates complications associated with the interpretation of data that might arise because of the different rates of IA formation by NadB when using oxygen as an electron acceptor as opposed to fumarate (*3*). Under both oxic and anoxic conditions, the NadA reaction displays clean Michealis−Menten behavior with respect to the varied substrate DHAP. Surprisingly, under oxic conditions, the $V_{max}/[E_T]$ of the reaction (19.2 ± 0.2 min^{-1}) is 12-fold greater than that observed under conditions that are both anoxic and reducing (1.6 ± 0.1 min^{-1}). Moreover, $V_{max}/[E_T]K_m$ for the reaction under oxic conditions (80 ± 0.1 min^{-1} mM^{-1}) is

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Table 1: Activities of wt and Variant NadA Proteins

	$V_{max}/[E_T]$ (min^{-1})		
	wt	C291S	C294S
NadA(ox) with DTT	1.6	1.1	2.4
NadA(ox) without DTT	5.8	0.62	0.99
NadA(red)	2.3	1.4	7.0
NadA(red) with O_2 (air)	5.3	0.42	0.55
NadA(red) with Trx(ox)	12	1.8	8.5
NadA(red) with C35A Trx	3.5	1.5	8.8
NadA(ox)	7.9	0.43	0.39
NadA(ox) with Trx(red)	1.6	1.6	0.90
NadA(ox) with C35A Trx	8.5	0.52	0.58

17-fold greater than that observed under anoxic and reducing conditions (4.7 ± 0.2 min^{-1} mM^{-1}).

The observation of O_2-dependent changes in NadA activity suggested redox regulation of the protein via oxidation of the Fe−S cluster or disulfide-bond formation, perhaps via Cys291 and Cys294. During incubation under oxidizing conditions (100 μM potassium ferricyanide), no evidence of a $[4Fe\text{-}4S]^{3+}$ cluster was observed by electron paramagnetic resonance spectroscopy; the protein remained largely diamagnetic but displayed a small amount (<10%) of a $[3Fe\text{-}4S]^{+}$ cluster (data not shown), suggesting that cluster oxidation is not responsible for the increase in activity. This observation is also consistent with the previous finding that the $[4Fe\text{-}4S]^{2+}$ cluster can be reduced to the +1 oxidation state (*7, 8*). To assess the effect of the redox state of cysteines 291 and 294 on NadA activity, each was changed individually to serine, which blocks potential disulfide-bond formation between the two amino acids. Under conditions that are both anoxic and reducing, the Cys291Ser and Cys294Ser variants exhibited $V_{max}/[E_T]$ values of 1.9 ± 0.1 and 6.3 ± 0.4 min^{-1} and $V_{max}/[E_T]K_m$ values of 2.2 ± 0.1 and 17 ± 0.2 min^{-1} mM^{-1}, respectively. Unlike the wild-type (wt) protein, the two variants were too unstable under oxic conditions for a complete steady-state analysis.

In a separate series of experiments, activity determinations for wt NadA and the Cys291Ser and Cys294Ser variants were performed under various conditions (Table 1). When wt NadA was pre-exposed to oxygen to allow formation of the putative disulfide bond, the $V_{max}/[E_T]$ of the enzyme upon incubation in the presence of 5 mM DTT (1.6 min^{-1}) was 3.6-fold lower than that obtained in the absence of DTT (5.8 min^{-1}). By contrast, the Cys291Ser and Cys294Ser variants exhibited opposite effects when treated similarly; their activities in the absence of DTT were lower, presumably because of their inability to form an intramolecular disulfide bond coupled with their increased instability (Table 1). Similar behavior was observed when NadA was prereduced by incubation with DTT and then subjected to gel filtration under anaerobic conditions to remove the reductant. The activity of the protein was 2.3-fold greater when determined under oxic conditions (air) than when determined under anoxic conditions. Again, the Cys291Ser and Cys294Ser variants exhibited opposite behavior; the activity of the Cys291Ser variant was slightly lower (3.3-fold) under oxic conditions than under anoxic conditions, while the activity of the Cys294Ser variant was significantly lower (13-fold).

Because thioredoxin (Trx) is known to be involved in reversible disulfide bond reduction in the cytosol (*11*), its ability to regulate turnover of *E. coli* NadA was assessed

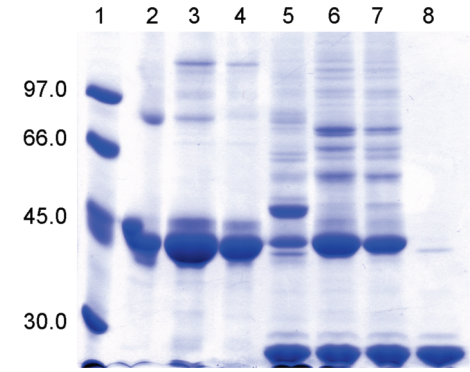

FIGURE 1: Cross-linking of Trx Cys35Ala with NadA via disulfide-bond formation: lane 1, molecular mass markers; lane 2, wt NadA; lane 3, NadA (C291S); lane 4, NadA (C294S); lane 5, wt NadA with Trx (C35A); lane 6, NadA (C291S) with Trx (C35A); lane 7, NadA (C294S) with Trx (C35A); and lane 8, Trx (C35A).

(Table 1). When prereduced NadA was added to an assay mixture containing excess oxidized thioredoxin, the $V_{max}/[E_T]$ of the enzyme was observed to increase 5.2-fold over that of the prereduced enzyme treated similarly but added to an assay mixture lacking oxidized Trx. By contrast, the activities of the Cys291Ser and Cys294Ser variants remained relatively constant. Similarly, when preoxidized wt NadA was added to an assay mixture containing reduced thioredoxin, its activity decreased 4.9-fold. Again, the Cys291Ser and Cys294Ser variants responded differently; their activities increased slightly, presumably because of the decreased lability of the cluster in the presence of a reductant. By contrast, the Cys35Ala Trx variant had little or no effect on the activity of wt NadA (Table 1), as did oxidized DTT (data not shown), suggesting that the redox potential of the Cys291−Cys294 disulfide bond is considerably more positive than −330 mV, the midpoint potential of DTT (*12*).

The reduction of disulfides by Trx takes place via a two-step process. The first step involves an intermolecular attack of Cys32, the more reactive cysteine, on the disulfide bond, resulting in a mixed-disulfide intermediate. Cleavage of the mixed disulfide occurs upon intramolecular attack of Cys35 on Cys32 (*13*). The mixed-disulfide intermediate does not accumulate with *E. coli* Trx, because the second step of the reaction is significantly more favorable than the first. However, use of a Trx Cys35Ser or Cys35Ala variant allows the mixed-disulfide intermediate to be isolated in significant quantities (*14*). In Figure 1, a nonreducing SDS−PAGE analysis of the interaction of preoxidized NadA with wt and variant forms of *E. coli* Trx is displayed. Preoxidized NadA migrates primarily as a monomer (molecular mass of 40 kDa), although a small amount (<10%) of dimer is observed (Figure 1, lane 2). Similar migratory properties are observed for the Cys291Ser and Cys294Ser variants (Figure 1, lanes 3 and 4). When oxidized wt NadA is treated with the Trx Cys35Ala variant, a shift in the molecular mass of NadA is observed that is consistent with the addition of 12 kDa, the approximate molecular mass of *E. coli* Trx (Figure 1, lane 5). By contrast, intermolecular cross-linking is not observed with the NadA Cys291Ser and Cys294Ser variants (Figure 1, lanes 6 and 7). Figure 1 shows that Trx dimer is generated under aerobic incubation via intermolecular disulfide formation, although most of the protein, which has exited the gel because of its small mass, is still monomeric. The appearance of small amounts of other bands at various molecular masses

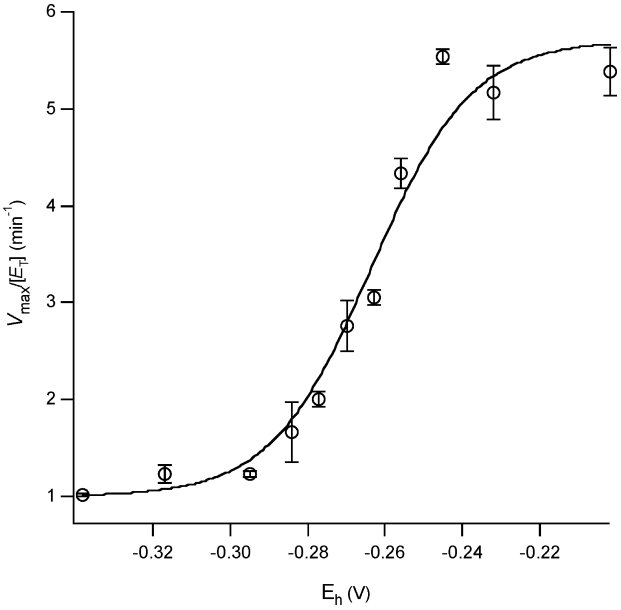

FIGURE 2: Determination of the midpoint potential of the disulfide bond in *E. coli* NadA. The solid line is a fit to eq 1, which assumes an obligate two-electron redox couple.

during aerobic incubation of NadA with the Trx variant suggests that dimeric Trx may be cleaved by one or more of the nine Cys residues found in *E. coli* NadA.

To approximate the midpoint potential of the Cys291−Cys294 disulfide bond, the activity of wt NadA was assessed in the presence of varying ratios of oxidized to reduced Trx, using the known midpoint potential of Trx to calculate the redox potential of the solution (E_h) at each $[Trx]_{ox}/[Trx]_{red}$ combination (Figure 2). Observed rates versus E_h were plotted and fitted to a modified form of the Nernst equation (eq 1)

$$V_{max}/[E_T] = k_1\{1 + 10^{[n(E-E_0)]0.059}\}^{-1} + k_2(1 - \{1 + 10^{[n(E-E_0)]0.059}\}^{-1}) \quad (1)$$

where k_1 and k_2 are k_{cat} values for oxidized and reduced NadA, respectively, n is the number of electrons transferred ($n = 2$), E is the applied potential, E_0 is the standard potential of the redox couple, and $V_{max}/[E_T]$ is the observed maximal velocity at a given applied potential (see the Appendix in the Supporting Information for derivation), affording a midpoint potential (E_0) of approximately −264 mV and rate constants k_1 and k_2 of 5.7 ± 0.2 and 0.9 ± 0.2, respectively.

The biosynthesis and recycling of NAD^+ and its derivatives is a highly regulated process (*1*), and the intracellular redox state of *E. coli*, as reflected by the ratio of NADH to NAD^+, varies as a function of the environmental redox state of the organism (*15*). During shifts from aerobic to anaerobic growth in several facultative bacteria, the total intracellular NADH concentration has been shown to remain constant, while the intracellular concentration of NAD^+ decreases rapidly. It was suggested that the regulation of NAD^+ synthesis is directly or indirectly influenced by oxygen (*16*). Our finding herein of a redox-active disulfide bond in *E. coli* NadA, which displays a midpoint potential (−264 mV) that is similar to that of *E. coli* Trx (−270 mV) (*17*) as well as that of the *E. coli* cytosol (−280 mV) (*18*), supports its physiological relevance in the regulation of NAD^+ biosynthesis and recycling.

SUPPORTING INFORMATION AVAILABLE

Materials and methods, Scheme S1, Figure S1, and the Appendix. This material is available free of charge via the Internet at http://pubs.acs.org.

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