The [4Fe–4S] cluster of quinolinate synthase from *Escherichia coli:* Investigation of cluster ligands

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Abstract Nicotinamide adenine dinucleotide (NAD) derives from quinolinic acid which is synthesized in *Escherichia coli* from L-aspartate and dihydroxyacetone phosphate through the concerted action of L-aspartate oxidase and the [4Fe–4S] quinolinate synthase (NadA). Here, we addressed the question of the identity of the cluster ligands. We performed in vivo complementation experiments as well as enzymatic, spectroscopic and structural in vitro studies using wild-type vs. Cys-to-Ala mutated NadA proteins. These studies reveal that only three cysteine residues, the conserved Cys113, Cys200 and Cys297, are ligands of the cluster. This result is in contrast to the previous proposal that pointed the three cysteines of the $C_{291}XXC_{294}XXC_{297}$ motif. Interestingly, we demonstrated that Cys291 and Cys294 form a disulfide bridge and are important for activity.
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1. Introduction

Nicotinamide adenine dinucleotide (NAD) plays a crucial role as a cofactor in numerous essential redox biological reactions. In fact, in all living organisms, NAD derives from quinolinic acid, the biosynthetic pathway of which differs among organisms [1–3]. In most eukaryotes and some bacteria, quinolinic acid is produced via the degradation of tryptophan whereas in *Escherichia coli* or *Salmonella typhimurium* it is synthesized from L-aspartate and dihydroxyacetone phosphate

(DHAP) as the result of the concerted action of two enzymes, the L-aspartate oxidase, a flavin adenine dinucleotide (FAD)-dependent flavoenzyme encoded by the L-aspartate oxidase (*nadB*) gene, and the quinolinate synthase, encoded by the quinolinate synthase (*nadA*) gene (Fig. 1) [1,4]. Besides the de novo synthesis of NAD, a salvage pathway exists that enables NAD to be recycled. This pathway usually proceeds via degradation of NAD to nicotinic acid (NA), followed by conversion of NA to nicotinic acid mononucleotide by nicotinic acid phosphoribosyltransferase (NAPRTase). *Mycobacterium tuberculosis,* a tubercular pathogen, and *Helicobacter pylori,* a major cause of gastroduodenal disease, were described to have a non-functional salvage pathway and thus cannot recycle NA to NAD. The presence of distinctly different pathways in most prokaryotes and eukaryotes for the biosynthesis of quinolinic acid and the absence of the salvage pathway for some suggests that NadA might prove to be a key target for the design of antibacterial agents.

Recently, NadA from *E. coli* was characterized as an [Fe–S] enzyme with a [4Fe–4S] cluster essential for its activity [5,6]. The [4Fe–4S] cluster proved to be very sensitive to oxygen. Indeed, when NadA is exposed to air, the $[4Fe–4S]^{2+}$ cluster is degraded into a $[2Fe–2S]^{2+}$ form and $S = 5/2$ species as observed by Mössbauer and EPR spectroscopy [6]. This is in agreement with previous in vivo observations that de novo NAD biosynthesis is a pathway sensitive to hyperbaric oxygen and that NadA is specifically the oxygen-sensitive site [7]. O_2 sensitivity has been observed in the case of [4Fe–4S] clusters in dehydratases such as aconitase or in "radical-SAM" enzymes [8,9]. All these enzymes have in common a [4Fe–4S] cluster ligated by only three cysteines whereas the fourth iron is coordinated by a non-protein ligand, the hydroxyl and carboxylate groups of the citrate substrate in the case of aconitase and the amino carboxylate group of the *S*-adenosylmethionine (SAM) moiety in the case of radical-SAM enzymes [9,10]. This specific arrangement is likely to be at the origin of the instability of the cluster. It was thus tempting to suggest that NadA also belongs to this class of [4Fe–4S] proteins and contains a cluster only ligated by three cysteines.

NadA from *E. coli* contains nine cysteine residues (Fig. 2) which can be classified as follows: three strictly conserved (Cys113, Cys200 and Cys297) and six partially conserved (Cys64, Cys119, Cys128, Cys195, Cys291 and Cys294) residues. Since NadA contains a CXXCXXC motif, it was previously proposed that the three cysteines of the $C_{291}XXC_{294}XXC_{297}$ motif were the iron ligands of the [4Fe–4S] cluster [7]. This

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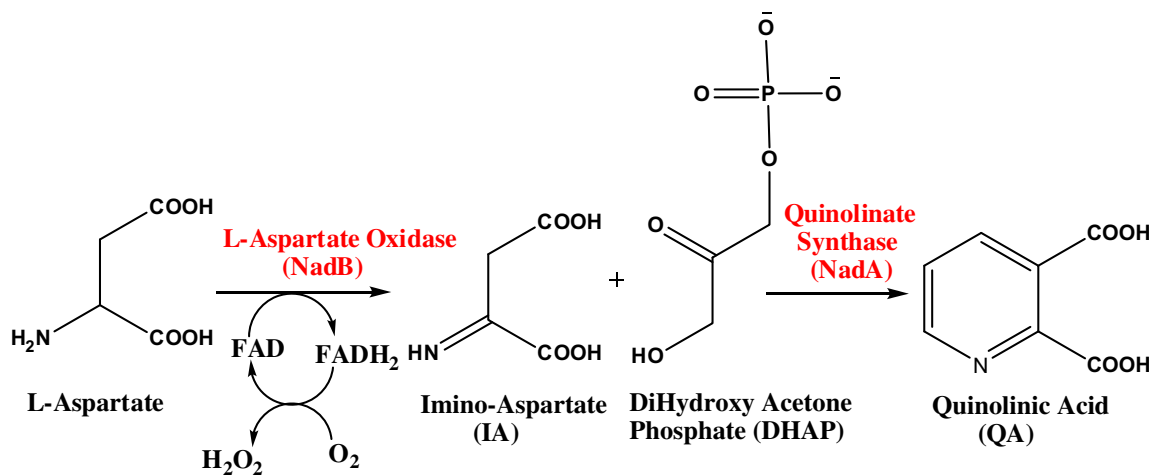*E-mail addresses:* mfontecave@cea.fr (M. Fontecave), sollagnier@cea.fr (S. Ollagnier de Choudens).

Fig. 1. Reaction catalyzed by the quinolinate synthase. Iminoaspartate (IA) is generated by the FAD-dependent L-aspartate oxidase enzyme (NadB). The quinolinate synthase NadA protein catalyzes its condensation with dihydroxy-acetonephosphate (DHAP) producing quinolinate. In vitro, fumarate is used as electron acceptor instead of dioxygen to regenerate FAD.

Fig. 2. Sequence alignment of NadA proteins from different organisms. ECOLI, *Escherichia coli*; MYCTU, *Mycobacterium tuberculosis*; VIBCH, *Vibrio cholerae*; HELPY, *Helicobacter pylori*; PYRHO, *Pyrococcus horikoshii*; BACSU, *Bacillus subtilis*; CLOAB, *Clostridium acetobutylicum*; LISIN, *Listeria innocua*; PSEAE, *Pseudomonas aeruginosa*; SALTY, *Salmonella typhimurium*; SHIFL, *Shigella flexneri*; YERPE, *Yersinia pestis*. Boxes represent conservation of cysteine residues within NadA sequences, (*) represents a full conserved amino-acid.

hypothesis has not been subjected to an experimental test. In order to solve this issue we performed in vivo complementation experiments as well as enzymatic, spectroscopic and structural in vitro studies using wild-type vs. Cys-to-Ala mutated NadA proteins. We demonstrated that (i) Cys113, Cys200 and Cys297 are ligands of the cluster and (ii) Cys291 and Cys294, which are not involved in the [Fe–S] coordination, form a disulfide bridge and are essential for in vivo activity. These results may provide important information helping to unravel the cat-

alytic mechanism and facilitating the design of antibacterial drugs.

2. Material and methods

2.1. Plasmids and strains

E. coli MG1655nadA mutant strain (Km^R) was constructed as already described [6]. E. coli C43(DE3) (Stratagene) was used to produce the recombinant protein NadA and the mutant forms. pET-NadB

Table 1
Oligonucleotides used as primers to generate the coding strands of mutated *nadA* genes

| WT | P1: 5′GTAAAACGAGATGGTCATATGAGCGTAATG3′ |
| | P2: 5′CCCCCAAAGCTTAACGTTACGCC3′ |

Amino acid substitution	Primer sequence
C113A	P1: 5′CTTCAGGCTGAA**GCT**TCACTGGATCTC3′
	P2: 5′GAGATCAGTGA**AGC**TTCAGCCTGAAG3′
C119A	P1: 5′GAATGTTCACTGGATCTCGGC**GCC**CCTGTTGAAGAATTTAAC3′
	P2: 5′GTTAAATTCTTCAACAGG**GGC**GCCGAGATCCAGTGAACATTC3′
C200A	P1: 5′GCTGGCAGGGTGCC**GCT**ATTGTGCATGATG3′
	P2: 5′CATCATGCACAAT**AGC**GGCACCCTGCCAGC3′
C291A	P1: 5′GGTGAGGGTGCAACC**GCC**CGCAGCTGCGCGCATTG3′
	P2: 5′CAATGCGCGCAGCTG**CGG**GCGGTTGCACCCTCACC3′
C294A	P1: 5′GCAACCTGCCGCAGC**GCC**CGCGCATTGTCCGTGG3′
	P2: 5′CCACGGACAATGCGC**GGC**GCTGCGGCAGGTTGC3′
C297A	P1: 5′CGCAGCTGCGCGCAT**GCT**CCGTGGATGGCCATG3′
	P2: 5′CATGGCCATCCACGG**AGC**ATGCGCGCAGCTGCG3′
C291A–C294A	P1: 5′GGTGAGGGTGCAACC**GCC**CGCAGC**GCC**CGCGCATTGTCCGTGG3′
	P2: 5′CCACGGACAATGCGC**GGC**GCTGCG**GGC**GGTTGCACCCTCACC3′
C294A–C297A	P1: 5′GCAACCTGCCGCAGC**GCC**CGCGCAT**GCT**CCGTGGATGGCCATG3′
	P2: 5′CATGGCCATCCACGG**AGC**ATGCGC**GGC**GCTGCGGCAGGTTGC3′
C291A–C294A–C297A	P1: 5′GGTGAGGGTGCAACC**GCC**CGCAGC**GCC**CGCGCAT**GCT**CCGTGGATGGCCATG3′
	P2: 5′CATGGCCATCCACGG**AGC**ATGCGC**GGC**GCTGCG**GGC**GGTTGCACCCTCACC3′

The substituted nucleotides are in bold.

plasmid allowing overexpression of the L-Asparate oxidase NadB protein was obtained as already described [6]. DH5α was used for *nadA* cloning.

2.2. Cloning and construction of wild-type and mutated NadA-overexpressing plasmids

The nadA-encoding gene was amplified by polymerase chain reaction using *E. coli* DH5α genomic DNA as a template. The following primers were used: 5′-gtaaaacgaggt*catatg*agcgtaatg-3′ (NdeI site underlined, ATG codon in uppercase) hybridized to the non-coding strand at the 5′ terminus of the gene and 5′-ccccc*aaagctt*aacgttacgcc-3′ (HindIII site underlined) hybridized to the coding strand. Polymerase chain reactions (PCRs) were run on a Stratagene RoboCycler Gradient 40 machine as follows: genomic DNA (50 µg) of *E. coli* was denatured for 4 min at 94 °C in the presence of the primers (5 µM each). The Pfu DNA polymerase (2.5 U) and deoxynucleotide mix (0.4 mM each) were added, and 20 cycles (30 s at 94 °C, 1 min at 55 °C and 2 min 30 s at 72 °C) were then performed followed by a 10-min elongation step at 72 °C. The PCR products were then digested with NdeI and HindIII and then ligated into a pT7-7 vector with an histidine tag digested by the same enzymes, yielding plasmid pT$_{7-7}$-NadA6H plasmid (Amp^R). *E. coli* nadA gene inserted into the expression vector pT$_{7-7}$ was submitted to directed mutagenesis, to change cysteine 113, 119, 200, 291, 294 and 297 into alanine, using the QuickChange Site-directed mutagenesis kit from Stratagene according to the manufacturer's instructions. The oligonucleotides used as mutagenic primers are described in Table 1. The mutant plasmids were entirely sequenced to ensure that no error was introduced during PCR reaction. The plasmids were then named pT$_{7-7}$-NadAC113A6H, pT$_{7-7}$-NadAC119A6H, pT$_{7-7}$-NadAC200A6H, pT$_{7-7}$-NadAC291A6H, pT$_{7-7}$-NadAC294A6H, pT$_{7-7}$-NadAC297A6H, pT$_{7-7}$-NadAC291A–C294A6H, pT$_{7-7}$-NadAC294A–C297A6H and pT$_{7-7}$-NadAC291A–C294A–C297A6H.

2.3. Expression and anaerobic purification of wild-type and mutated NadA proteins

E. coli competent C43(DE3) strain were transformed with pT$_{7-7}$-NadA6H, pT$_{7-7}$-C113A6H, pT$_{7-7}$-C119A6H, pT$_{7-7}$-C200A6H, pT$_{7-7}$-C291A6H, pT$_{7-7}$-C294A6H, pT$_{7-7}$-C297A6H, pT$_{7-7}$-C291A–C294A6H, pT$_{7-7}$-C294A–C297A6H and pT$_{7-7}$-C291A–C294A–C297A6H vectors. Cells were grown at 37 °C in LB medium containing 100 µg/ml amplicillin to an OD at 600 nm of 0.5 and expression was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) (Eurogentec) for 3 h at 37 °C. The bacterial pellet obtained from an aerobic culture were resuspended into a glove box (Jacomex B553 (NMT)) in degassed buffer A (100 mM Tris–HCl, 50 mM NaCl, pH 7.5) containing 0.6 mg/ml lysozyme and 1 mM PMSF, then transferred into

ultracentrifuge tubes. The solution was frozen quickly (outside the glove box) and thawed (inside the glove box). This procedure was repeated three times and followed by an ultracentrifugation (4 °C, 45 000 rpm, 90 min). The clear supernatant solution was loaded anaerobically into a Ni-NTA column (5 ml) equilibrated with buffer A. After an extensive washing (0.2 l of buffer A) NadA was eluted with buffer A containing 0.16 M imidazole. Pure fractions were concentrated and stored at −80 °C.

2.4. Aggregation state analysis

FPLC gel filtration with an analytical Superdex-200 (Pharmacia Amersham Biotech) at a flow rate of 0.5 ml/min equilibrated with buffer B (100 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM dithiothreitol (DTT)) was used for size determination of oligomerization state of NadA mutants. A gel filtration calibration kit (calibration protein II, Boehringer Inc.) was used as molecular weight standards.

2.5. NadA enzymatic activity

NadA enzymatic activity was assayed under anaerobic conditions, inside a glove box at 37 °C, and the time-dependent formation of quinolinic acid (QA) was measured directly by HPLC. The assay contained in a final volume of 1 ml, buffer C (50 mM Na–HEPES, 100 mM KCl, pH 7.5), 2 mM DHAP, 33 mM L-aspartate, 25 mM fumarate, 6.1 µM of either wild-type or mutant as-isolated proteins. After a 5 min pre-incubation of the assay mixture at 37 °C the reaction was initiated by the addition of 7.1 µM FAD-containing NadB. For the quantification of quinolinic acid, aliquots (100 µl) were removed at different times and added to 5 µl 2 N H$_2$SO$_4$ to quench the reaction. Precipitated proteins were removed by centrifugation at 15000 rpm for 15 min. Fifty microliters of the supernatant was injected directly onto the HPLC. The QA produced was analyzed and quantified by a HP-1100 HPLC system after injection onto a Tosoh TSK Gel ODS-120T (4.6 mm × 15 cm) column. The column was eluted with buffer D (0.03% TFA, pH 2.4) at a flow rate of 0.5 ml/min. QA was detected by its absorbance at 260 nm and was eluted with a retention time of 9.1 min. After 20 min of elution the column was regenerated by applying a linear gradient from 0% to 100% acetonitrile in 0.03% TFA at 0.5 ml/min over 10 min and re-equilibrated before injection of the next sample. Authentic quinolinic acid (Sigma, St. Louis, MO) was used to generate a standard curve from 0 to 40 nmol.

2.6. Complementation experiments

E. coli MG1655nadA mutant strain (ΔNadA) which has a disruption in the *nadA* gene encoding quinolinate synthase [6] was transformed with PT$_{7-7}$ plasmid or PT$_{7-7}$ plasmids that contained *nadA* or *nadA*$_{C113A}$, *nadA*$_{C119A}$, *nadA*$_{C200A}$, *nadA*$_{C291A}$, *nadA*$_{C294A}$ and
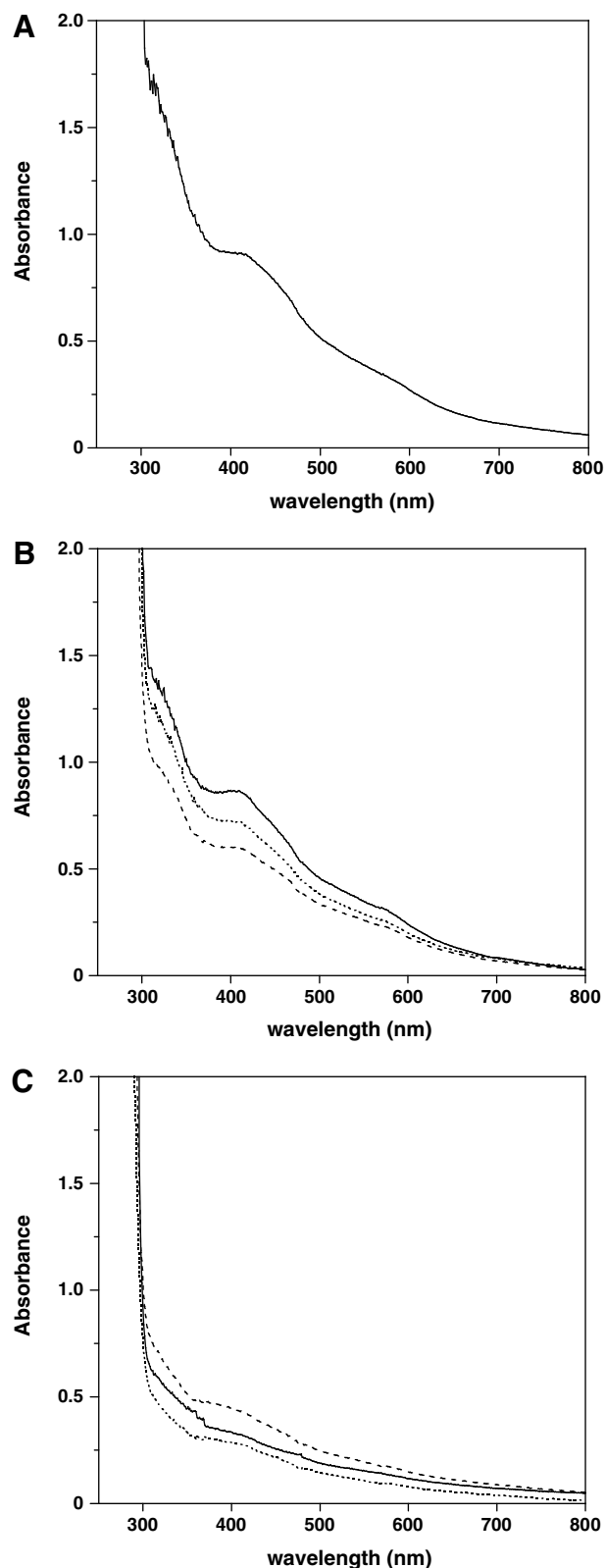
Fig. 3. UV–visible spectrum of anaerobically as-isolated NadA WT (A) and mutant proteins (B and C). (A) 60 μM, (B) 65 μM (—) C294A, 70 μM (···) C291A, 53 μM (- - - -) C119A and (C) 52 μM (—) C297A, 51 μM (····) C200A, 55 μM (- - - -) C113A.

*nadA*_{C297A} genes allowing the overexpression of the wild-type or the corresponding mutated proteins, respectively. These transformed

strains were isolated on M9 minimal medium supplemented with 0.4% glucose, 0.1% casein, 0.0001% thiamine, 2 mM MgCl₂, with or without 12.5 μg/ml NA. Growth was controlled at 37 °C after 12–16 h.

2.7. Preparation of the reduced quinolinate synthase

The quinolinate synthase (145 μM) was incubated with thioredoxin (TRX) (1 μM), thioredoxin reductase (TRR) (0.23 μM) and reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (0.3 mM) in Buffer E (0.1 M Tris–HCl, 50 mM KCl, pH 8). Oxidation of NADPH was monitored at 340 nm inside the glove box by UV–visible spectroscopy. Alternatively, the reduced NadA protein was prepared inside the box by 1 h incubation with (0–50 mM) DTT following by a desalting step and assayed for quinolinate synthase activity.

2.8. Analysis

Protein concentrations were measured by the method of Bradford using bovine serum albumin as a standard that in the case of NadA and NadB overestimates the concentration by a factor of 2.45, as determined by the quantitative amino acid analysis of purified NadA and NadB [11]. Protein-bound iron was determined under reducing conditions with bathophenanthroline disulfonate after acid denaturation of the protein [12] and labile sulfide by the method of Beinert [13].

2.9. UV–visible spectroscopy

UV–visible spectra were recorded with a Cary 1 Bio (Varian) spectrophotometer.

3. Results

3.1. Purification of NadA mutant proteins

The different mutated plasmids were prepared from plasmid pT₇₋₇-NadA6H using a polymerase chain reaction-based method (see Section 2). The mutated enzymes, in which one of the six cysteine residues, namely Cys113, Cys119, Cys200, Cys291, Cys294 and Cys297, was changed into alanine, were overexpressed in *E. coli* C43(DE3) under conditions similar to the wild-type [6]. Double mutants, namely NadAC291A–C294A and NadAC294A–C297A, and one triple mutant, namely NadAC291A–C294A–C297A, were also expressed under the same conditions. As judged from SDS–PAGE analysis of whole cells and soluble extracts, the levels of expression for all mutants were as for the wild-type enzyme (data not shown). Since they all contain a tag of six histidines at the N-terminus they were purified using a Ni-NTA column that specifically retains proteins containing a cluster of histidines as already described

Table 2
Characterization of NadA proteins (wild-type and mutants) for their metal content, specific activity and oligomerization state

	Color	Fe and S content	AS (nmol QA/min/mg)	Oligomerization state
WT	+	4	56	M
C113A	−	1.0	0	O
C119A	+	2.9	30	M
C200A	−	0.9	0	O
C291A	+	3.9	5.2	M
C294A	+	3.2	3.1	M
C297A	−	0.4	0	O
C291A–C294A	+	3.7	6	M
C294A–C297A	−	0.6	0	/
C291A–C294A–C297A	−	0.5	0	/

M, monomer; O, oligomers; (/), not determined; (+), brown color; (−), colorless.

[6,14]. In order to avoid oxidation of the [Fe–S] cluster the purification was performed anaerobically (from the extraction to the last chromatography step). Each mutant was obtained with a relatively good purity (between 70% and 90%) as checked by SDS–PAGE, according to the standard procedures developed for the wild-type (data not shown). For each culture we obtained between 1 and 5 mg protein per liter of culture. The purified proteins were subjected to chromatography on a Superdex-200 column. The elution profiles were identical to the wild-type for NadAC119A, NadA291A, NadAC294A and NadAC291A–C294A mutant proteins which behave as a monomer with an apparent mass of 40 000 Da (theoretical mass of the wild-type: 39 305 Da calculated from the sequence). In contrast, NadAC113A, NadAC200A and NadAC297 proteins behave as a mixture of oligomers, from dimer to tetramer.

3.2. Biochemical and spectroscopic characterization of NadA mutants

The purified wild-type protein has a red-brownish color, displays a light absorption spectrum with a characteristic band at 420 nm (Fig. 3A) and contains 4.2 iron and 4.0 sulfide/polypeptide chain in agreement with the presence of a [4Fe–4S] cluster per polypeptide chain as already described in the literature (Table 2) [5,6]. From the absorption spectrum and the metal content analysis it is clear that mutants can be classified in two groups. The first group, including NadAC119A, NadAC291A and NadAC294A, displays similar properties to the wild-type NadA protein and contains between 2.9 and 3.9 iron and sulfide/polypeptide chain (Fig. 3B and Table 2). Interestingly, the NadAC291A–C294A double mutant contains 3.6 iron and sulfide/polypeptide chain. Furthermore, the stability of the cluster in this protein was not affected by the double mutation (data not shown). In contrast, a single mutation at position Cys113, Cys200 and Cys297 has a drastic impact on the spectroscopic properties and metal content of NadA. Indeed, the corresponding NadAC113A, NadAC200A and NadAC297A proteins, which defined the second group, are almost colorless in agreement with the absence of absorption bands in UV–visible spectroscopy and contain only between 0.4 and 1 iron and sulfur atom/polypeptide chain (Fig. 3C and Table 2). Not surprisingly, the same results were obtained for NadAC291A–C297A and NadAC291A–C294A–C297A double and triple mutant proteins, respectively, with a maximum of 0.6 iron and sulfide/protein for both (Table 2). These results suggest that the cluster is coordinated by the three cysteine Cys113, Cys200 and Cys297 residues only and not by the cysteine Cysl19, Cys291 and Cys294 residues.

4. Enzymatic activity of NadA mutants

The purified mutated proteins were then analyzed for their ability to form quinolinic acid in vitro from DHAP and imino-aspartate in the presence of NadB as described [14]. As already mentioned, this reaction requires the presence of the [4Fe–4S] cluster within NadA protein. The results are summarized in Table 2. As expected from the UV–visible spectrum and the iron and sulfur content, NadAC113A, NadAC200A and NadAC297A as well as NadAC294A–C297A and NadAC291A–C294A–C297A proteins were inactive supporting the

conclusion that the cluster is coordinated by the corresponding Cys113, Cys200 and Cys297 cysteine residues. In contrast, NadAC119A, NadAC291A, NadAC294A and NadAC291A–C294A proteins, which can assemble a cluster (Fig. 3B and Table 2), display quinolinate synthase activity. However, they were significantly less active than the wild-type NadA protein (from 5% to 53% of wild-type activity).

4.1. Cys113, Cys200, Cys291, Cys294 and Cys297 are required for complementation of an E. coli ΔNadA strain

An E. coli MG1655 ΔnadA strain lacking an active nadA gene exhibits NAD auxotrophy [6,15]. NAD biosynthesis can be restored by two ways: either bringing plasmidic copies of nadA in the nadA mutant strain or by supplementing the medium with NA. Indeed, NA can be metabolized by the bacteria into NAD allowing growth [1]. The functionality of the different cysteines was assayed in vivo using the complementation experiment of the E. coli MG1655 ΔnadA strain. First, control experiments were carried out using the vector PT$_{7-7}$ with no nadA gene insert and the vector pT7-NadA for expression of the wild-type NadA. After one night at 37 °C only bacteria transformed with pT$_{7-7}$-NadA were able to grow without NA in the medium (Table 3) showing that NadA produced under these conditions is functional. Then, E. coli MG1655 ΔnadA strain was transformed with each of the plasmids containing the nadA gene in which one of the cysteine residues (Cys113, Cys119, Cys200, Cys291, Cys294 and Cys297) has been changed into alanine. As expected and shown in Table 3, E. coli MG1655 ΔnadA transformed with pT$_{7-7}$-NadAC113A, pT$_{7-7}$-NadAC200A and pT$_{7-7}$-NadA297A was unable to produce NAD and thus to grow without NA. These mutants do not assemble the [4Fe–4S] cluster and the in vivo result provides strong evidence that these residues are required for activity likely through chelation of the cluster. Surprisingly, two additional cysteine residues, Cys291 and Cys294, are required for in vivo activity. Finally, Cys119 mutation had no effect on NAD biosynthesis since the ΔnadA strain, complemented with pT$_{7-7}$-NadAC119A was able to grow on minimal medium without NA (Table 3). All these results showed that five cysteine residues are required for full activity in vivo, Cys113, Cys200, Cys291, Cys294 and Cys297.

Table 3
Complementation experiments

	Growth on M9 + NA	Growth on M9 − NA
pT$_{7-7}$	+	−
pT$_{7-7}$-NadA	+	+
pT$_{7-7}$-NadAC113A	+	−
pT$_{7-7}$-NadAC119A	+	+
pT$_{7-7}$-NadAC200A	+	−
pT$_{7-7}$-NadAC291A	+	−
pT$_{7-7}$-NadAC294A	+	−
pT$_{7-7}$-NadAC297A	+	−

Escherichia coli MG1655nadA mutant strain (ΔNadA) was transformed with pT$_{7-7}$ plasmid or pT$_{7-7}$ plasmids that contained nadA or nad$_{C113A}$, nadA$_{C119A}$, nadA$_{C200A}$, nadA$_{C291A}$, nadA$_{C294A}$ and nadA$_{C297A}$ genes. These transformed strains were isolated on M9 minimal medium supplemented with or without 12.5 μg/ml nicotinic acid (NA). Growth (+ or −) was controlled at 37 °C after 12–16 h.
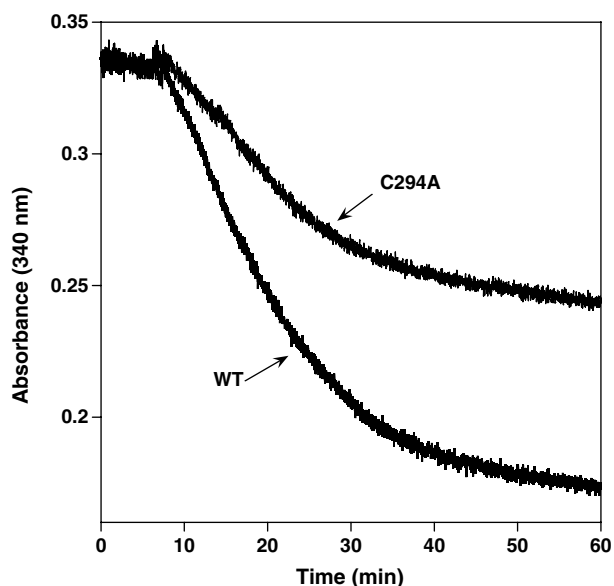
Fig. 4. TRX-mediated oxidation of NADPH by NadA protein. In a 1-mm optical path UV–visible cuvette, the NadA protein (145 µM), wild-type (lower trace) or C294A mutant (upper trace), was incubated inside the glove box with 0.3 mM NADPH and 0.23 µM TRR in 0.1 M Tris–HCl, pH 8. After 8 min, 1 µM thioredoxin (TRX) was added and the reaction was allowed to proceed for 50 min. The C291A protein as well as the C291A–C294A double mutant protein display the same NADPH oxidation rate and kinetic than C294A mutant (data not shown).

4.2. Cys291 and Cys294 are involved in a disulfide bridge

Five cysteine residues are required for activity, but only three are involved in cluster coordination namely Cys113, Cys200 and Cys297. The CXXC motif involving Cys291 and Cys294 is found in a number of redox active proteins such as thioredoxin and thiol-disulphide oxidoreductase family [16]. We tested the ability of Cys291 and Cys294 to form an in-tra-molecular disulfide bridge. In Fig. 4 is shown the time-dependent decrease of the absorbance of NADPH (0.3 mM) at 340 nm when a solution of the NadA protein was complemented inside the glove box first with TRR (0.23 µM) and after 5 min with TRX (1 µM). Control experiments done without NadA showed a negligible decrease of the absorbance at 340 nm compared with that seen in Fig. 4 with wild-type NadA. Quantitative analysis of these data showed that the reaction was almost completed after 50 min when about 274 µM of NADPH has been converted to NADP$^+$. This amount matched twice the concentration of the NadA protein (145 µM) indicating that the NadA underwent a four-electron reduction and thus carries two disulfide groups amenable to reduction. When NadAC291A or NadAC294A proteins (145 µM) or NadAC291A–C294A (130 µM) were used instead of wild-type NadA we observed a decrease in the absorption at 340 nm corresponding to a half-reduction of the wild-type (consumption of 156 µM and 139 µM of NADPH for NadA-C291A and NadAC294A proteins, respectively, and consumption of 118 µM of NADPH for NadAC291A–C294A protein) showing that NadAC291A and NadAC294A proteins are together involved in one disulfide group (Fig. 4). That the disulfide bridge is important for activity was then checked by assaying the wild-type quinolinate synthase activity after reduction. For that purpose, NadA was treated with the thio-

redoxin/thioredoxin reductase/NADPH system for 60 min and then assayed for quinolinate formation as described in the experimental part. A 80% decrease of activity was observed under these conditions (data not shown). A similar result was obtained when NadA was preincubated with 1 mM DTT, a dithiol reducing agent (data not shown). We checked that under these conditions the decrease of activity was not due to a lack or degradation of metal center since the protein still contains 90% of its iron and sulfur content after DTT treatment. These results further confirm that NadA activity is dependent on the presence of a disulfide group on the protein involving C291 and C294.

5. Discussion

NadA, the quinolinate synthase from *E. coli*, contains a [4Fe–4S] cluster which was shown to be essential for activity [5,6]. Here, we address the question of the ligands of the iron–sulfur cluster. This knowledge was important to understand whether the chemical properties of this cluster were reflecting a novel coordination environment and help us to understand the role of the cluster in the mechanism.

We have clearly established that the [4Fe–4S] cluster is coordinated by only three cysteine residues, the conserved Cys113, Cys200 and Cys297, based on the four following facts.

First, anaerobic purification of the three NadA mutants, NadAC113A, NadAC200A and NadAC297A led to proteins which displayed a pale color and a poor metal content with regard to wild-type and the other mutants that contain roughly 3–4 iron and sulfide/polypeptide chain. Second, site directed mutagenesis of these cysteines generated totally inactive proteins during in vitro quinolinate formation in the presence of NadB, L-aspartate, DHAP and fumarate. Third, plasmids containing *nadA* gene in which one of these cysteine residues (Cys113, Cys200 or Cys297) has been changed to alanine were unable to complement an *E. coli nadA* knock-out in minimal medium in the absence of nicotinic acid. Fourth, in contrast to wild-type and the other mutants which behaved as a monomer in solution, C113A, C200A and C297A proteins existed as oligomers. The lack of the cluster and the availability of reactive free cysteine residues may be at the origin of disulfide bridges and therefore inactive oligomers. Finally, the finding that NadAC291A–C294A mutant still assembles a cluster, in contrary to NadAC291A–C297A and NadAC291A–C294A–C297A proteins, further demonstrate that Cys291 and Cys294 are not involved in the iron–sulfur coordination and that only Cys113, Cys200 and Cys297 coordinate the cluster. In general, such clusters with only three cysteine residues as ligands are prone to oxidative degradation into [3Fe–4S] and [2Fe–2S] clusters. The presence of a $S = 1/2$ [3Fe–4S]$^{+1}$ cluster, accounting for 30–40% of total iron, was observed by EPR spectroscopy in the case of wild-type NadA protein which was exposed to air during 10 min (data not shown) and the presence of [2Fe–2S]$^{2+}$ was already observed by Mossbauer spectroscopy after longer exposure to air [6]. Considering that it is extremely rare to observe [3Fe–4S]$^{+1}$ species from a [4Fe–4S] with four cysteine ligands this result further support a NadA cluster with an accessible and reactive Fe site.

A coordination by only three cysteine residues in the case of NadA was already proposed by Gardner and Fridovich based

on NadA amino-acid sequence and the specific oxygen sensitivity of the enzyme in the extracts [7]. Even though the cluster was not characterized, they proposed Cys291, Cys294 and Cys297 as the iron ligands since these residues take part of a CXXCXXC motif, known to generally provide ligands to [4Fe–4S] clusters in numerous enzymes [17]. However, based on a more careful analysis of cysteine residues in NadA sequences from a variety of organisms, paralleled with in vivo experiments and characterization of the in vitro properties of the cysteine-to-alanine mutants, we demonstrate here that this proposal is wrong. This provides an illustration of the difficulty to identify cysteine ligands only on the basis of their relative position in primary amino-acids sequences. In the case of [Fe–S] proteins, whereas some cysteine motifs are widely used to chelate the cluster, there is an increasing occurrence of clusters chelated by cysteines spread all over the entire sequence. NadA is one such example, even though it also contains a CXXCXXC motif. One has thus to be extremely cautious about consensus motifs which come out from primary sequences and a systematic analysis of fully conserved amino-acids should be done.

In general, iron atoms of [4Fe–4S] clusters are coordinated by the sulfur atom of four cysteinyl residues. However, there are notable exceptions and reported precedents of iron–sulfur centers with only three cysteine ligands. Aconitase is probably the most extensively studied example of such proteins [9]. In this case, the fourth ligand is a solvent hydroxyl in the absence of the substrate and two oxygen atoms from the substrate when it is protein-bound. Radical-SAM enzymes also contain a [4Fe–4S] with only three cysteine residues as ligands, the fourth one being the SAM cofactor [18]. Ni–Fe hydrogenase and 4-hydroxybutyryl-CoA dehydratase contain a [4Fe–4S] with one coordinating histidine residue at the fourth position [19,20]. Finally, in the [4Fe–4S] center of a ferredoxin from *P. furiosus*, one of the iron sites has an aspartate ligand in place of a cysteine [21], whereas the dihydroxypyrimidine dehydrogenase iron–sulfur cluster is maintained within the protein through coordination by an oxygen atom from a glutamine residue in that case [22]. Histidine, aspartate or glutamine residues are thus possible candidates for playing the role of the fourth ligand in the quinolinate synthase. Aspartate 67, Aspartate 178, Glutatmate 228 and Histidine 226 in *E. coli* are conserved among the NadA proteins and thus might be potential candidates (Fig. 2). However, as discussed below, we favour a situation more similar to aconitase or radical-SAM enzymes, with an iron site occupied by a solvent molecule which can be displaced by a substrate molecule. Indeed, the presence of an accessible iron site provides the cluster with specific chemical reactivity. In the case of the radical-SAM enzymes the cluster is endowed with a redox-catalytic activity leading to the reductive cleavage of SAM into methionine and a radical species. In the case of aconitase, the Lewis acidity of the iron site is used to increase the electrophilicity of the substrate and thus facilitate the reaction. The finding that only three cysteines coordinate the cluster in the case of NadA opens the question of the role of the cluster in the formation of quinolinic acid. Two mechanisms for the formation of quinolinic acid were proposed both involving two dehydration steps [1,23]. Therefore, one can speculate that the NadA cluster plays a catalytic role similar to that of aconitase and hydrolyase enzymes [24].

Interestingly, our experiments revealed that Cys291 and Cys294 residues are important for both in vivo and in vitro activity. From the experiments showing that NadA wild-type is able to oxidize two equivalent of TRX whereas NadAC291A and NadAC294A single mutant proteins oxidize only one equivalent it is likely that Cys291 and Cys294 are involved in a disulfide bridge. We exclude that each of these two cysteines form a disulfide bridge with one other partially conserved cysteine residues, namely Cys64, Cys128 and Cys195, for the following reasons: (i) the $C_{291}XXC_{294}$ motif is a typical sequence for disulfide bridge formation and (ii) the NadAC291A–C294A double mutant oxidizes one equivalent of TRX, like NadAC291A and NadAC294A single mutants, whereas one would expect zero if each of the corresponding cysteine would have been involved independently in a disulfide with another cysteine residue (Cys64, Cys128 or Cys195). The proximity of these cysteine residues (Cys291 and Cys294) with regard to one of the ligands of the cluster (Cys297) and thus to the cluster might suggest that they are located in an important part of the polypeptide chain critical for folding of the protein. However, preliminary results seem to exclude an effect of the Cys291 Cys294 pair on the folding of the protein and on the stability of the cluster. On the other hand, we cannot rule out a redox active role of these cysteines during catalysis, even though we have no evidence for such an hypothesis. On the contrary to Cys291 and Cys294, Cys119 is not important for activity as shown by in vivo experiments. The slightly lower activity observed in vitro with regard to wild-type (53%) might be related to its close proximity to Cys113, one of the ligands of the cluster.

In conclusion, we have demonstrated that the [4Fe–4S] of NadA is coordinated by only three cysteine residues, the conserved Cys113, Cys200 and Cys297 residues. The role of the cluster in the catalytic mechanism as well as that of the cysteines residues of the CX2C motif are the next issues to address in the laboratory.

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