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Eukaryotic NAD$^+$ Synthetase Qns1 Contains an Essential, Obligate Intramolecular Thiol Glutamine Amidotransferase Domain Related to Nitrilase*

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NAD$^+$ is an essential co-enzyme for redox reactions and is consumed in lysine deacetylation and poly(ADP-ribosyl)ation. NAD$^+$ synthetase catalyzes the final step in NAD$^+$ synthesis in the well characterized *de novo*, salvage, and import pathways. It has been long known that eukaryotic NAD$^+$ synthetases use glutamine to amidate nicotinic acid adenine dinucleotide while many purified prokaryotic NAD$^+$ synthetases are ammonia-dependent. Earlier, we discovered that glutamine-dependent NAD$^+$ synthetases contain N-terminal domains that are members of the nitrilase superfamily and hypothesized that these domains function as glutamine amidotransferases for the associated synthetases. Here we show yeast glutamine-dependent NAD$^+$ synthetase Qns1 requires both the nitrilase-related active-site residues and the NAD$^+$ synthetase active-site residues for function *in vivo*. Despite failure to complement the lethal phenotype of *qns1* disruption, the former mutants retain ammonia-dependent NAD$^+$ synthetase activity *in vitro*, whereas the latter mutants retain basal glutaminase activity. Moreover, the two classes of mutants fail to *trans*-complement despite forming a stable heteromultimer *in vivo*. These data indicate that the nitrilase-related domain in Qns1 is the fourth independently evolved glutamine amidotransferase domain to have been identified in nature and that glutamine-dependence is an obligate phenomenon involving intramolecular transfer of ammonia over a predicted distance of 46 Å from one active site to another within Qns1 monomers.

Nicotinamide-adenine dinucleotide (NAD$^+$)[1] and its phosphorylated form NADP are essential for oxidizing reactions in the cell, whereas reduced forms of these co-enzymes, NADH and NADPH, are essential for supplying reducing equivalents. Beyond the reversible functions of NAD$^+$ in hundreds of enzyme-dependent redox reactions, at least two types of enzymes consume NAD$^+$ in eukaryotic nuclei in the process of forming or reversing post-translational modifications. Poly(ADP-ribose) polymerases respond to DNA strand breaks by transferring the ADP-ribose moiety of NAD$^+$ to target proteins, thereby facilitating DNA repair and other processes (1, 2). Sirtuins, a family of protein lysine-deacetylases related to yeast Sir2, reverse regulatory acetyl modification of lysines on histones, p53, and other proteins typically to alter the assembly of nucleoprotein complexes (3, 4). NAD$^+$ is consumed by Sir2 to produce a mixture of 2′- and 3′-*O*-acetylated ADP-ribose plus nicotinamide and the deacetylated polypeptide (5). NAD$^+$-dependent deacetylation reactions are required not only for alterations in gene expression but also for repression of ribosomal DNA recombination and extension of lifespan in response to calorie restriction (6, 7). In prokaryotes, Sirtuins perform lysine deacetylation to alter chromatin structure (8) as well as to regulate acetyl-CoA synthetase and potentially other metabolic enzymes (9).

Interest in the biological functions of Sir2 and Sirtuins has lead to re-examination of the biosynthetic routes to NAD$^+$ in yeast and animals. It has long been established that the *de novo*, salvage, and import routes to NAD$^+$ converge on nicotinic acid mononucleotide, which is then adenylylated to nicotinic acid dinucleotide (NaAD or deamido-NAD). Working with fungal and animal extracts, Preiss and Handler (10, 11) determined that the final enzyme in NAD$^+$ biosynthesis, NAD$^+$ synthetase, uses glutamine as an amide donor as depicted in Scheme 1.

$$\text{NaAD} + \text{ATP} + \text{glutamine} \rightarrow \text{NAD}^+ + \text{AMP} + \text{PPi} + \text{glutamate}$$

SCHEME 1

Surprisingly, purification of NAD$^+$ synthetase from *Escherichia coli* established the first of several prokaryotic NAD$^+$ synthetases to be ammonia-dependent, at least *in vitro* (12). Not long thereafter, purified yeast NAD$^+$ synthetase was shown to be a large multimer and evidence was presented for the reaction pathway shown in Fig. 1 in which the enzyme forms an activated NaAD-adenylate (NaAD-AMP) intermediate that is subsequently exchanged for the side chain amine group from glutamine (13). This work also showed that yeast NAD$^+$ synthetase can use ammonia *in vitro* at elevated pH (13). However, the pH/activity profile of yeast NAD$^+$ synthetase suggests that the enzyme does not deprotonate NH_4^+, which predominates at neutral pH. Thus, the glutamine dependence of eukaryotic (10, 11) *versus* the ammonia dependence of *E. coli* (12) NAD$^+$ synthetases created a problem in biochemistry and evolutionary biology that has stood for over 35 years.

In the mean time, scores of enzymes have been characterized that couple a glutaminase domain to an ammonia-utilizing domain (14). These coupled glutaminases, termed glutamine

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[1] The abbreviations used are: NAD$^+$, nicotinamide adenine dinucleotide; NaAD or deamido-NAD, nicotinic acid adenine dinucleotide; GAT, glutamine amidotransferase; Qns1, glutamine-dependent NAD synthetase; 5FOA, 5-fluoroorotoic acid; HA, hemagglutinin.

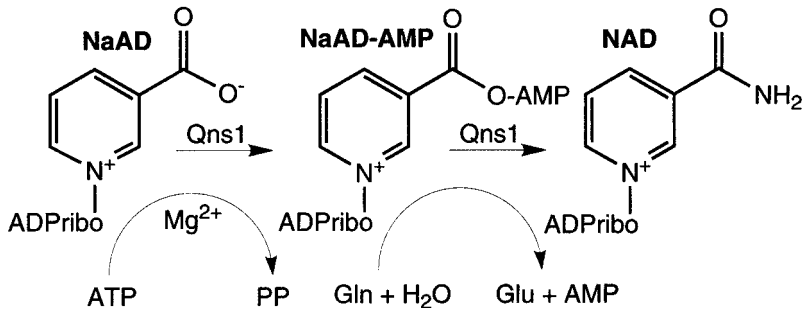

FIG. 1. **Reaction scheme of glutamine-dependent NAD$^+$ synthetase.** Qns1 catalyzes ATP-Mg^{2+}-dependent adenylylation of NaAD to form NaAD-AMP. In the second step, glutamine is consumed to glutamate and the released ammonia attacks the adenylylated carbon, releasing AMP and forming NAD$^+$.

amidotransferase (GAT) domains, have been classified as members of the triad-type or the N-terminal nucleophile-type superfamilies (14). More recently, a superfamily of aminoacyl-tRNA *trans*-amidases were described as a third unrelated type of GAT (15). Here we show that a domain related to nitrilase (16) containing a conserved Glu-Lys-Cys catalytic triad (16) is the long sought GAT domain for eukaryotic NAD$^+$ synthetase, Qns1 (Q for glutamine-dependent, NAD$^+$ synthetase). As we noted earlier (16), the nitrilase-related domain is found in all of the eukaryotic NAD$^+$ synthetases and those prokaryotic NAD$^+$ synthetases such as that from *Mycobacterium tuberculosis* that are glutamine-dependent (17). Our data establish that functional GAT and NAD$^+$ synthetase active sites must be in the same polypeptide to function *in vivo* and *in vitro*. By showing that a heteromultimer consisting of polypeptides with mutations in the two different active sites is stably formed and yet is inactive *in vivo*, we provide genetic evidence for an obligate intramolecular path for ammonia. According to a homology model constructed for the glutaminase and the NAD$^+$ synthetase domains of Qns1, the channel for transit of ammonia from the glutaminase active site to NaAD is ~46-Å long and is lined with 12 amino acids that have been conserved in all of the known eukaryotic NAD$^+$ synthetases.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The *QNS1* gene containing its native promoter was amplified by PCR with oligonucleotide primers R003 and R004 using *Saccharomyces cerevisiae* strain SEY6210 (18) genomic DNA as a template. The *Bam*H1 and *Eco*R1-restricted PCR product was cloned into vector pRS416 (19) to generate plasmid pB175. The sequenced insert from pB175 was transferred to vector pRS413 (19) to generate pB177. Using single-stranded plasmid pB177 as template, primers 7039, 7040, 5095, 7033, and 7032 were used to generate *qns1*-E45A, *qns1*-K114A, *qns1-C175A*, *qns1-D365A*, and *qns1-E527A* alleles in plasmids pB379, pB378, pB178, pB252, and pB251, respectively (20). The *qns1-E527A* insert from plasmid pB251 was moved to vector pRS414 (19) to generate plasmid pB338. Allele-specific N-terminal 3xFLAG-*QNS1* fusions were created by PCR amplification of the *QNS1* alleles from plasmids pB177, pB178, and pB251 with primers 7119 and 7120. Products of these reactions were used as templates for PCR with primers 7118 and 7120, restricted with *Bam*HI and *Eco*RI, and cloned into vectors p413*GAL1* and p414*GAL1* (21), respectively, to generate plasmids pB320, pB326, and pB324. Allele-specific 2 × HA-*QNS1* fusions were similarly made from plasmids pB177 and pB251 as templates, primers 7117 and 7120 in the first PCR and primers 7116 and 7120 in the second PCR. Restricted products were cloned into vectors p413*GAL1* and p414*GAL1* (21), respectively, to generate pB321 and pB325. Plasmids pB341, pB342, and pB343 were created by side-directed mutagenesis of plasmid pB326 with a mixture of primers 7039 and 7040 to create *E45A* and/or *K114A* mutations in addition to the *C175A* mutation of plasmid pB326. To clone the open reading frame annotated as ammonia-dependent NAD$^+$ synthetase from *Thermotoga maritima* (22) (GenBank™ AE001780, locus AE001780_12), which actually encodes glutamine-dependent NAD$^+$ synthetase (16), primers 5189 and 5168 were used to amplify a specific product from plasmid clone BTMIN90 from the American Type Culture Collection. The *Bam*HI and *Xho*I-restricted product was then inserted into vector p414*GAL1* (21) to generate plasmid pB229. To create bacterial expression plasmid pB337, the *QNS1* open reading frame obtained by PCR with primers 5093 and 5094 was cloned into the *Nde*I and *Xho*I sites of vector pSGA04 (23). Expression plasmids for *QNS1* mutants, plasmids pB339, pB340, pB358, and pB359, were created by mutagenesis of plasmid pB337 with primers 5095, 7032, 7039, and 7040. All of the constructs were confirmed by DNA sequencing. Plasmids are summarized in Table I. Primer sequences are provided in Table II.

Yeast Strain Constructions—To generate a fragment for disruption of *QNS1*, primers R001 and R002 were used to amplify the *kanMX4* marker of pRS400 (24). Diploid yeast strain BY127 of genotype *MATa*/*MATα*, *ura3*/*ura3*, *trp1*/*trp1*, *his3*/*his3*, *leu2*/*leu2*, *LYS2*/*lys2*, *MET15*/*met15* was integratively transformed with the resulting product. Stable transformants were selected on YPD medium supplemented with 0.4 mg/ml G418 and tested for heterozygosity at the *QNS1* locus by PCR with primers R003 and R004. Heterozygous strain BY165 was transformed with plasmid pB175 carrying *S. cerevisiae QNS1* and was induced to sporulate. Haploids obtained after tetrad dissection of this strain were scored for mating type, lysine and methionine prototrophy, and sensitivity to 1 mg/ml 5-fluoroorotic (5FOA). Strain BY165–1d of genotype ***MATa***, *qns1*Δ::*kanMX4, ura3*, *trp1*, *his3*, *leu2*, *met15* carrying pB175 was transformed with plasmid pB229 carrying *T. maritima QNS1* driven by a *GAL1* promoter. These transformants exhibited galactose-dependent 5FOA resistance and then galactose-dependent and glucose-inhibited growth. Standard yeast media and methods were as described previously (25).

Enzyme Purification and Analysis—His-tagged yeast Qns1 proteins were expressed and purified from 2-liter cultures of *E. coli* strain BL21(DE3) grown at 30 °C to an optical density (600 nm) of 0.4, induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside, and aerated an additional 5 h. Cells were lysed by sonication in 150 mM NaCl, 1 mM imidazole, 20 mM potassium phosphate, pH 7.5. Clarified lysate was loaded on a 2-ml nickel-nitrilotriacetic acid column, which was washed with 50 ml of 150 mM NaCl, 1 mM imidazole, 5% glycerol, and 20 mM potassium phosphate, pH 7.5. Enzymes were eluted with 150 mM NaCl, 200 mM imidazole, 20 mM potassium phosphate, pH 7.5, and 5% glycerol and concentrated to 14–24 mg/ml in 150 mM NaCl, and 20 mM Tris Cl, pH 7.5. NAD$^+$ synthetase (13) assays were performed with 1 mM NaAD, 2 mM ATP, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.0, 56 mM KCl, 0.2 mg/ml bovine serum albumin, and either 2 mM NH_4Cl or 20 mM glutamine. Glutaminase assays were performed with the same ingredients with or without NaAD. Ammonia-dependent NAD$^+$ synthetase assays (0.1 ml) were initiated with 2.7 μg of wild-type or mutant enzymes. Glutamine-dependent assays were initiated with 270 ng of wild-type or 9 μg of mutant Qns1 enzymes. Glutamine-dependent assay volumes were 0.15 ml to allow one-third of the sample to be assayed for glutamate production. NaAD-free glutaminase assays contained 9 μg of enzyme in 0.05 ml. After triplicate 60-min, 37 °C incubations, reactions were terminated by heating at 100 °C for 2 min, chilled on ice, and centrifuged for 10 min at 12,000 rpm. Supernatants were taken for product determinations using either alcohol dehydrogenase or glutamate dehydrogenase-coupled assays to determine NAD$^+$ and glutamate (26) formation, respectively. NAD$^+$ assays were in 0.9 ml of 10 mM sodium pyrophosphate, 0.1% ethanol. Absorbance (340 nm) was measured before and after a 10-min room temperature incubation with 39 units of alcohol dehydrogenase. NAD$^+$ standards (0–100 nmol) were measured in the same reaction conditions before and after alcohol dehydrogenase incubation. Glutamate assays were in 1 ml of 50 mM Tris HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, 0.5 mM NAD$^+$, and 2.2 units glutamate dehydrogenase. Reactions were incubated for 90 min at 37 °C and clarified with a 2-min centrifugation at 12,000 rpm. For glutamate determinations, glutamate standards (0–32 nmol) underwent both incubations in parallel with samples and contained all of the ingredients other than Qns1. Absorbance (340 nm) was measured to quantify NAD$^+$ reduced by glutamate dehydrogenase, which is limited by glutamate production.

Immunoprecipitation—Wild-type laboratory yeast strain SEY6210

TABLE I
Plasmids used in this study

Name	Vector	Insert	Promoter	Marker
pB175	pRS416	*QNS1*	*QNS1*	*URA3*
pB177	pRS413	*QNS1*	*QNS1*	*HIS3*
pB178	pRS413	*qns1-C175A*	*QNS1*	*HIS3*
pB229	p414*GAL1*	*T. maritima QNS1*	*GAL1*	*TRP1*
pB251	pRS413	*qns1-E527A*	*QNS1*	*HIS3*
pB252	pRS413	*qns1-D365A*	*QNS1*	*HIS3*
pB320	p413*GAL1*	*3xFLAG-qns1*	*GAL1*	*HIS3*
pB321	p414*GAL1*	*2xHA-QNS1*	*GAL1*	*TRP1*
pB324	p413*GAL1*	*3xFLAG-qns1-E527A*	*GAL1*	*HIS3*
pB325	p414*GAL1*	*2xHA-qns1-E527A*	*GAL1*	*TRP1*
pB326	p413*GAL1*	*3xFLAG-qns1-C175A*	*GAL1*	*HIS3*
pB327	p414*GAL1*	*2HA-qns1-C175A*	*GAL1*	*TRP1*
pB337	pSGA04	*His7-QNS1*	T7	*bla*
pB338	pRS414	*qns1-E527A*	*QNS1*	*TRP1*
pB339	pSGA04	*His7-qns1-C175A*	T7	*bla*
pB340	pSGA04	*His7-qns1-E527A*	T7	*bla*
pB341	p413*GAL1*	*3xFLAG-qns1-E45A, K114A, C175A*	*GAL1*	*HIS3*
pB342	p413*GAL1*	*3xFLAG-qns1-K114A, C175A*	*GAL1*	*HIS3*
pB343	p413*GAL1*	*3xFLAG-qns1-E45A, C175A*	*GAL1*	*HIS3*
pB358	pSGA04	*His7-qns1-E45A*	T7	*bla*
pB359	pSGA04	*His7-qns1-K114A*	T7	*bla*
pB378	pRS413	*qns1-K114A*	*QNS1*	*HIS3*
pB379	pRS413	*qns1-E45A*	*QNS1*	*HIS3*

TABLE II
Oligodeoxynucleotides used in this study (5′-3′)

R001	ATGTCACATCTTATCACTTTAGCTACATGCAACTTGAATCAATGGGCCCTAGATTGTACTGAGAGTGCAC
R002	CGTCATCCCCATATCTATCTCATCCGATTGAACGTAATCTTTAGTCATAGCTGTGCGGTATTTCACACCG
R003	ACGC**GGATCC**GAACTCTTTCAGTCAGTTAGATCCAT
R004	TCCG**GAATTC**GGAATGTAGTAATCAAGACTAATTGG
5093	CTCCCTGCCA**CATATG**TCACATCTTATCACTTTAGC
5094	ACTTCT**CTCGAG**CTAATCAATAGACATAATGTC
5095	TGTAAACAATTCTTCTGCAGTTTCTGTACCAAT
5118	GATAAGATGTGA**CATATG**TGGCAGGGAGTCAAA
5168	GATCCG**CTCGAG**TCAAAGAGGTTCTTTAAATCTG
5189	GTCGC**GGATCC**ATGAAAAGACTGAGAGTGACAC
7032	ATACCCACGTAAGCACGCATCAACATTTGCGCT
7033	TGCAGTTGCACAAGAGGCAATGCCCCCAGATAA
7039	GCCAGTTATTTCCAGTGCTGGGCCGACACGTAA
7040	ATTAGCTAACCAAATCGCAGGTCTTATGAACAA
7116	CGC**GGATCC**ATGTACCCATACGACGTACCTGATTACGCATATCGGTATGATGTGC
7117	ACGCATATCCGTATGATGTGCCGGACTATGCTTCACATCTTATCACTTTAGC
7118	CGC**GGATCC**ATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGACTAC
7119	AAGATCATGACATCGACTACAAAGACGATGACGACAAGTCACATCTTATCACTTTAGC
7120	CCG**GAATTC**CTAATCAATAGACATAATGTCAAGCGTTG
7121	CGC**GGATCC**ATGCATCATCACCATCACCATCATCTTATCACTTTAGCTACATGCAAC

(18) transformed with plasmids carrying different Qns1 alleles under *GAL1* promoter control were grown to early stationary phase in 50 ml of selective media with galactose as a carbon source. Cells were lysed by agitation with glass beads in 1 ml of phosphate-buffered saline with a protease inhibitor mixture (Roche Applied Science). Clarified lysates (0.5 ml) were incubated with 30 μl of a suspension of anti-FLAG antibody cross-linked to agarose beads. After a 60-min incubation, beads were washed five times with phosphate-buffered saline and captured proteins were extracted with 8 M urea and subjected to SDS-PAGE. Duplicate Western blots were performed by probing with 1:10,000 dilutions of anti-FLAG or anti-HA monoclonal antibodies followed by a 1:10,000 dilution of goat anti-mouse IgG-horseradish peroxidase conjugate. Immune complexes were detected with chemiluminescent peroxidase substrate (Sigma).

RESULTS AND DISCUSSION

QNS1 Is an Essential Yeast Gene—On the basis of its N-terminal domain of similarity to the nitrilase superfamily and its C-terminal similarity to single domain bacterial NAD$^+$ synthetases, we identified the YHR074W gene of *S. cerevisiae* as the apparent yeast glutamine-dependent NAD$^+$ synthetase (16), *QNS1*. This gene was disrupted with a G418 resistance marker in a wild-type diploid strain, which was induced to undergo meiosis and sporulation and was subjected to tetrad dissection. As shown in Fig. 2*A*, viability segregated 2 to 2. All of the viable progeny from this dissection were G418-sensitive, indicating that *QNS1* is either essential for spore germination or viability in *S. cerevisiae*.

The *QNS1* gene from *S. cerevisiae* and the homologous gene from the thermophilic eubacterium *T. maritima* were cloned into yeast centromeric plasmids, the latter gene under transcriptional control of the *GAL1* promoter. Transformation of the former *URA3*-based plasmid into *qns1* heterozygous strain PB165 prior to tetrad dissection allowed as many as four viable progeny to be recovered per tetrad (Fig. 2*B*). Transformation of the *T. maritima QNS1* plasmid into an isolate with *qns1*Δ covered by *S. cerevisiae QNS1* allowed the curing of the yeast gene, producing isolates that exhibited galactose-dependent and glucose-repressible growth. Fig. 2*C* shows this rare case of complementation of a eukaryotic gene deletion by a corresponding thermophilic gene. In contrast to its annotation as ammonia-dependent NAD$^+$ synthetase (22), this bacterial NAD$^+$ synthetase includes a nitrilase-related domain (16) and does indeed encode glutamine-dependent enzyme activity.[2] Because 5FOA-resistant, *i.e.* Ura- isolates (27) of strain PB165, could not be

[2] P. Bieganowski, H. C. Pace, and C. Brenner, unpublished results.

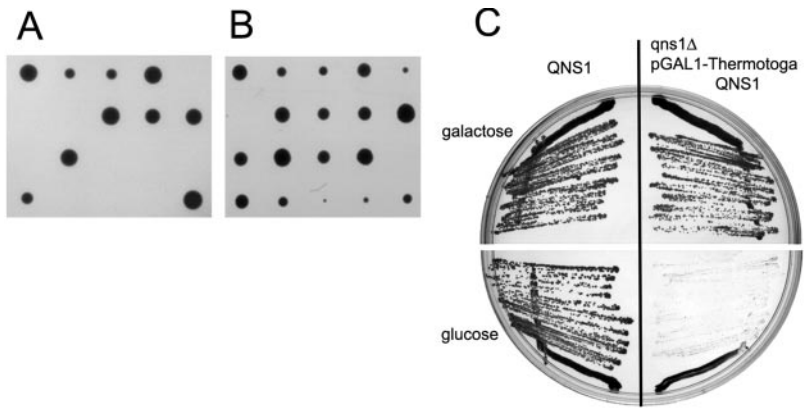

FIG. 2. ***QNS1* is an essential gene that can be complemented by the two-domain *QNS1* homolog from *T. maritima*.** *A*, yeast strain BY165 of genotype *MAT***a***/MATα*, *QNS1/qns1*Δ::*kanMX4* was sporulated and dissected. The two viable progeny per tetrad are G418-sensitive. *B*, yeast strain BY165 transformed with plasmid pB175 carrying *S. cerevisiae QNS1* produced tetrads with as many as four viable spores. *C*, G418-resistant isolate BY165–1d carrying plasmid pB175 was subjected to a plasmid shuffle to exchange for pB229, containing *QNS1* from *T. maritima* driven by the *GAL1* promoter. The resulting isolate exhibits galactose-dependent and glucose-inhibited growth.

recovered without a second Qns1-encoding plasmid and because transformants with the *T. maritima* plasmid exhibited galactose-dependent growth, we conclude that *QNS1* is an essential gene not only for spore germination but for cell growth.

Glutamine Dependent NAD$^+$ Synthetase Contains an Essential GAT Domain Related to Nitrilase—According to the domain structure of Qns1 (Fig. 3*A*), we predicted that the first 310 amino acids constitute a glutaminase domain containing an active-site Cys-175 that aligns with the essential nucleophile for all of the enzymes in the nitrilase superfamily (16). Additionally, according to the crystal structure of NitFhit and subsequent analysis, all of the members of the nitrilase superfamily contain a catalytic triad consisting of an absolutely conserved Glu and Lys in addition to the Cys nucleophile (16, 28, 29). This predicted ion pair corresponds to Glu-45 and Lys-114 of Qns1. Residues 311 to the C terminus of Qns1 align with single domain ammonia-dependent NAD$^+$ synthetases, such as those from *E. coli* and *Bacillus subtilis*. Based on crystal structures of *B. subtilis* NAD$^+$ synthetase (30, 31), we targeted Asp-365 and Glu-527 for substitution with Ala. Because the corresponding residues, Asp-50 and Glu-162 in *B. subtilis* NAD$^+$ synthetase, are involved in recognition of one of two Mg^{2+} ions positioned at the site of adenylylation/amidation of NaAD (30, 31), we reasoned that resulting Qns1 mutants would fail to form the NaAD-AMP intermediate and result in loss of function. Indeed, as shown in Fig. 3*B*, neither the E45A, the K114A, nor the C175A-predicted glutaminase mutants were functional *in vivo* nor were the predicted synthetase alleles D365A and E527A able to support growth. Additionally, truncation mutants missing either of the two domains failed to complement (data not shown). Thus, yeast NAD$^+$ synthetase is an obligate glutamine-dependent enzyme *in vivo*.

To test whether the point mutants were loss-of-function for the predicted reasons, we expressed and purified His-tagged wild-type and mutant Qns1 proteins in *E. coli*. For each protein, we measured glutamine-dependent NAD$^+$ synthetase activity, ammonia-dependent NAD$^+$ synthetase activity, and glutaminase activity in the presence and absence of NaAD (Table III). Consistent with the specific activity of native yeast glutamine-dependent NAD synthetase (13), recombinant Qns1 purified from *E. coli* produced 2440 ± 220 nmol NAD$^+$/min/mg and produced 3430 ± 180 nmol glutamate/min/mg, indicating that a single glutamine is hydrolyzed for each ammonia consumed by the NAD$^+$ synthetase active site. When glutaminase activity of wild-type enzyme was assayed in the absence of NaAD, glutamate production was reduced to 4% of the rate found in complete reactions. At pH 8, using NH_4Cl as the source of ammonia, wild-type Qns1 produced 4% as much NAD$^+$ as it did with glutamine as the ammonia source. Consistent with our prediction that the nitrilase-related domain is a GAT for eukaryotic NAD$^+$ synthetase (16), Qns1-C175A had at least a 5000-fold reduction in glutamine-dependent NAD$^+$ synthetase activity and a >250-fold reduction in glutaminase activity that was not further reduced by leaving out NaAD. Comparing the Qns1-C175A mutant to wild-type Qns1, there was a <2-fold reduction in ammonia-dependent NAD$^+$ synthetase activity, indicating that ammonia-dependent NAD$^+$ synthetase activity does not depend on a functioning GAT domain. Although the Qns1-E45A and Qns1-K114A mutants failed to complement the lethal phenotype of *qns1* deletion, we were able to measure 0.5 and 0.1% of the wild-type specific activity in glutamine-dependent NAD$^+$ synthetase activity (the latter of which was at least eight times higher than the background of the assay) and 6 and 3%, respectively, in glutaminase assays containing NaAD. Just as the C175A mutant lost the wild-type enzyme's 27-fold stimulation of its glutaminase active site with presence of NaAD, the glutaminase activity of the E45A mutant with the ammonia-accepting NaAD substrate was no higher than without NaAD. In contrast, the residual glutaminase activity measured for Qns1-K114A was stimulated by NaAD by >60-fold.

Although the near-normal ammonia-dependent NAD$^+$ synthetase activities of glutaminase mutants and the magnitude of the C175A mutant on glutamine-dependent NAD$^+$ synthetase activity were satisfying, the residual glutaminase activities of glutaminase active-site mutants were somewhat surprising. Therefore, we tested whether single, double, and triple glutaminase active-site mutants could function *in vivo* when overexpressed. Indeed, as shown in Fig. 3*C*, whereas none of these mutants retain sufficient function to complement when not overexpressed, the triple *qns1-E45A, K114A, C175A* mutant allows colony formation when overexpressed. These data corroborate the residual glutaminase activities measured *in vitro*. In addition, the data indicate that for survival-level glutamine-dependent NAD$^+$ synthetase function, a little glutaminase activity goes a long way. Secondly, the wild-type enzyme is not, to use Smith and Zalkin's term, "wasteful" of glutamine (32). As with glutamine phosporibosylpyrophosphate amidotransferase, engagement of the ammonia-accepting substrate (in this case, NaAD) activates the glutaminase activity of wild-type enzyme. Finally, mutation of the glutaminase domain has little or no effect on ammonia-dependent NAD$^+$ synthetase formation.

In contradistinction, the designed NAD$^+$ synthetase mutant, Qns1-E527A, eliminated all of the detectable NAD$^+$ production from either glutamine or ammonia, and having no ability to amidate NaAD, it exhibited no stimulation of its glutaminase activity by NaAD. However, this enzyme did retain basal glutaminase activity, performing in glutaminase assays with and without NaAD 60% as well as did the wild-type enzyme assayed without NaAD. Because Qns1-C175A specifically lost glutaminase activity and retained ammonia-dependent NAD$^+$

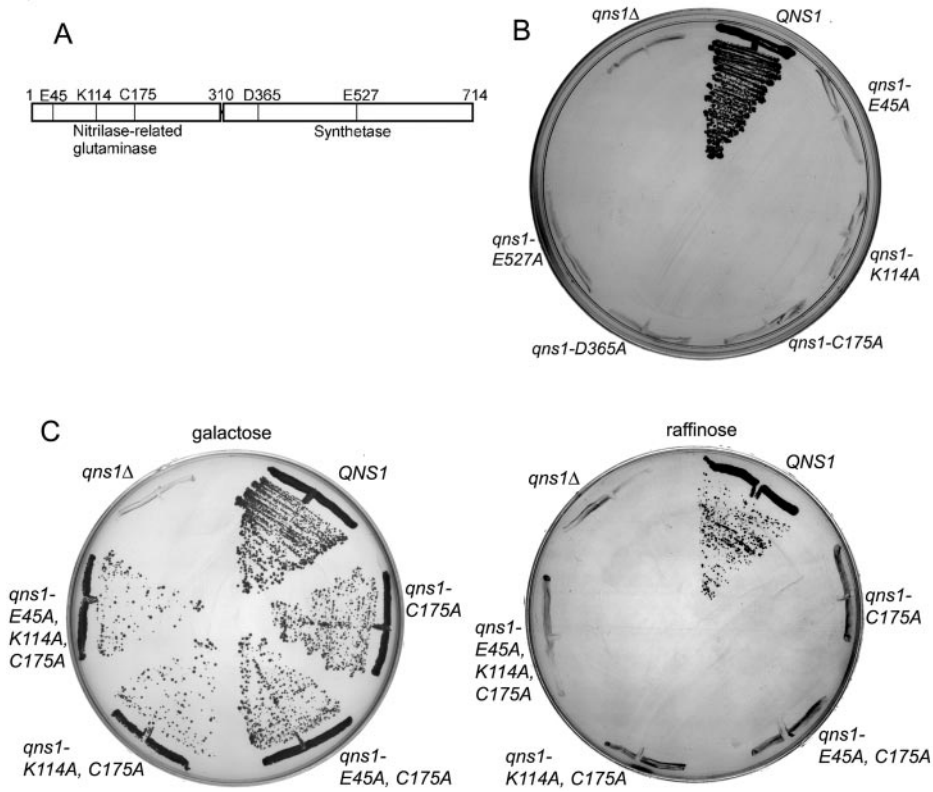

FIG. 3. **Qns1 contains two essential active sites for NAD$^+$ synthetase function *in vivo*.** *A*, domain structure of Qns1. The glutamine amidotransferase domain contains nitrilase-related active-site residues Glu-45, Lys-114, and Cys-175, whereas the NAD$^+$ synthetase domain contains an active site including Asp-365 and Glu-527. *B*, mutants targeting the catalytic triad of glutaminase, namely *qns1-E45A* (pB379), *qns1-K114A* (pB378), and *qns1-C175A* (pB178), and mutants targeting NAD$^+$ synthetase active-site residues, namely *qns1-D365A* (pB252) and *qns1-E527A* (pB251), fail to confer 5FOA resistance to BY165 (pB175) and are therefore nonfunctional *in vivo*. All of the constructs were expressed from the *QNS1* promoter and assayed on glucose media. *C*, to test whether the residual *in vitro* glutaminase activities of glutaminase domain mutants presented in Table III were biologically meaningful, single, double, and triple glutaminase mutants were expressed from the *GAL1* promoter, such that they would be expressed on raffinose and overexpressed on galactose. Clockwise, the transformants of strain BY165 (pB175) contained *QNS1* (pB320), *qns1-C175A* (pB326), *qns1-E45A, C175A* (pB343), *qns1-K114A, C175A* (pB342), and *qns1-E45A, K114A, C175A* (pB341). Although the 5FOA-curing frequencies of BY165 (pB175) were reduced as active-site residues were mutated, overexpression of even the triple mutant allowed colonies to support growth, indicating that residual glutaminase activities of glutaminase active-site mutants are real.

TABLE III
Active site-dependent enzymatic activities of recombinant qns1

Enzyme	Specific activity			
	Gln-dependent NAD synthetase	NH_3-dependent NAD synthetase	Glutaminase with NaAD	Glutaminase without NaAD
	nmol/min/mg			
Qns1	2870 ± 20	125 ± 3	4080 ± 30	151 ± 4
Qns1-E45A	13.2 ± 1.9	57.2 ± 1.0	267 ± 6	240 ± 2
Qns1-K114A	4.1 ± 0.5	107 ± 1	128 ± 4	10.9 ± 0.3
Qns1-C175A	< 0.5	76.7 ± 2.4	14.1 ± 2.4	34.3 ± 0.9
Qns1-E527A	< 0.5	<0.9	101 ± 5	118 ± 3

synthetase activity while Qns1-E527A lost NAD$^+$ synthetase activity and retained basal glutaminase activity, the data establish that both domains are essential *in vivo* and *in vitro* for the anticipated reasons. Thus, the nitrilase-related glutaminase domain is the fourth independently evolved GAT domain found in nature. Independently, the same conclusion was drawn from the heteromultimeric glutamine-dependent NAD$^+$ synthetase of *Thermus thermophilus* in which an NAD$^+$ synthetase subunit and a nitrilase-related glutaminase subunit are encoded by two genes in an operon.[3] Additionally, the human glutamine-dependent NAD synthetase was assayed *in vitro* before and after mutation of Cys-175, leading to identification of the nitrilase-related domain as a glutaminase (33).

[3] K. Shatalin and A. Osterman, personal communication.

A Genetically Defined Intramolecular Path for Ammonia—All of the known members of the nitrilase superfamily are dimers or larger and are frequently tetrameric (29). NAD$^+$ synthetase from *B. subtilis* is dimeric (30), and the yeast NAD$^+$ synthetase was reported to have a native size of greater than 600 kDa (13), although it would not be surprising if it looked like NitFhit (28), *i.e.* consisting of a central nitrilase-related tetramers linked to two NAD synthetase dimers. In multidomain and multimeric proteins, it is not unusual to find intragenic complementation by alleles that inactivate different domains (34). Given the potential complementarity of the two mutants, we wished to test whether co-expression of the two alleles would reconstitute glutamine-dependent NAD$^+$ synthetase. As shown in Fig. 4, *left panel*, no such complementation was observed. Failure to complement might have been the result of failure to express either of the enzymes or failure to form a heteromultimer containing both singly mutated proteins. To address these concerns, we tested by co-immunoprecipitation whether a heteromultimer of the two individually mutated proteins was assembled in cells. We expressed FLAG-tagged copies of wild-type and Qns1-C175A and Qns1-E527A mutants alone and in combination with HA-tagged wild-type and mutant Qns1 proteins. Cells were lysed, and proteins immunoprecipitated by anti-FLAG antibody beads were electrophoresed and probed for HA and FLAG epitopes. As shown in Fig. 4, *right panel*, anti-FLAG immunoprecipitates from cells expressing only an HA-tagged Qns1 construct produced no

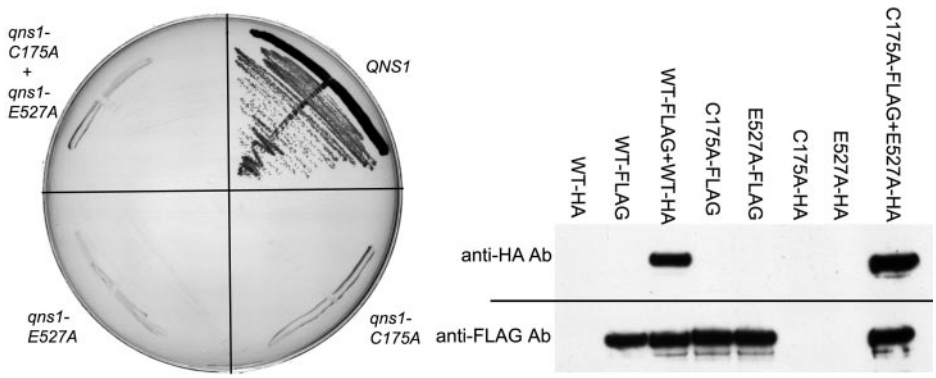

FIG. 4. **Genetic evidence for specific intramolecular paths for ammonia within Qns1 multimers.** *Left panel*, co-expression of glutaminase mutant Qns1-C175A and NAD$^+$ synthetase mutant Qns1-E527A fails to result in *trans*-complementation. *Right panel*, wild-type (*WT*) and mutant Qns1 enzymes were epitope-tagged as indicated and expressed alone and in combinations. Immunoprecipitation was performed with anti-FLAG-agarose beads. Samples were analyzed by Western blotting with anti-FLAG and anti-HA antibodies (*Ab*), showing that both mutants are expressed well and form a heteromultimer.

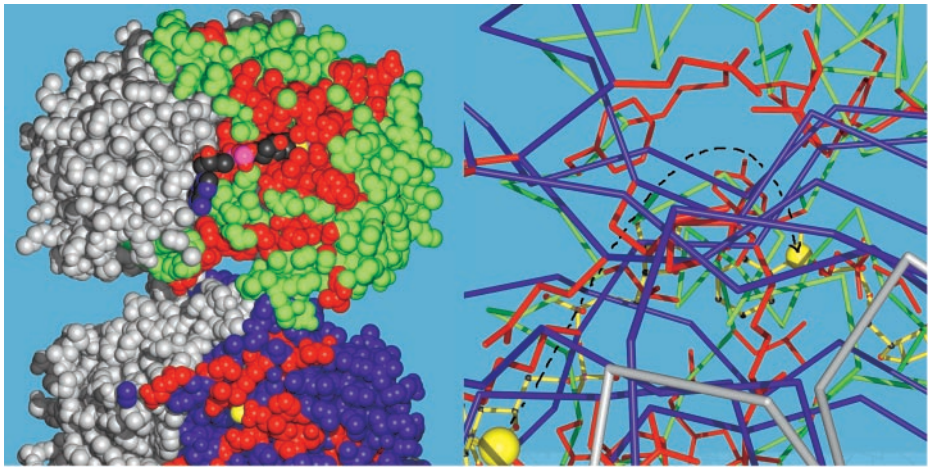

FIG. 5. **Threading and sequence conservation locate 45-Å paths for ammonia.** The glutaminase domain of Qns1 was threaded against the 15% identical Nit tetrameric core domain of NitFhit. The NAD$^+$ synthetase domain of Qns1 was threaded against the 25% identical dimeric ammonia-dependent NAD$^+$ synthetase from *B. subtilis* bound to the crystallographically determined NaAD-AMP intermediate (31). Pairs of Nit-related C termini projected toward pairs of NAD$^+$ synthetase N termini and suggested the overall organization of the domains. A glutaminase domain is in *blue*, and its connecting NAD$^+$ synthetase domain is in *green*. Residues that are identical in all of the known eukaryotic NAD$^+$ synthetases are *red*. A symmetry-related polypeptide is in *gray*. The active-site cysteine in the glutaminase domain and the nicotinic acid β carbon in NaAD-AMP are *colored yellow*. *Left side*, half of the predicted Qns1 tetramer is shown in a space-filling model in a slab view that cuts into the interior of the molecule to show much of the predicted route for ammonia. The *lower yellow sphere* marks the γ thiol group of Cys-175. The *upper yellow sphere* marks the β carbon of NaAD-AMP, ~45 Å away from Cys-175. *Right side*, the predicted Qns1 tetramer is rotated to place Cys-175 in the foreground at *lower left* and the β carbon of NaAD-AMP in the background. The apparent path for ammonia, lined by 12 residues conserved in all of the known eukaryotic NAD$^+$ synthetases, is shown in a *dashed line*.

reaction with anti-HA or anti-FLAG antibody, demonstrating that only specifically immunoprecipitated proteins are detected on the Western blot. Cells with FLAG-tagged Qns1 produced the expected sized anti-FLAG antigen and resulted in no spurious anti-HA reactivity. Cells expressing Qns1-C175A and Qns1-E527A produced Qns1 protein of the same size and abundance as those expressing tagged wild-type protein, indicating that the mutations did not significantly destabilize the enzyme. Co-expression of each of the epitope-tagged wild-type Qns1 proteins resulted in both constructs being immunoprecipitated by FLAG-tagged Qns1, thereby validating the assay for heteromultimerization. Finally, co-expression of HA-tagged Qns1-E527A with FLAG-tagged Qns1-C175A showed that these non-complementing mutants do, nonetheless, heteromultimerize. Because cells expressing the heteromultimer remain dependent on wild-type Qns1 for survival, we conclude that ammonia released from one polypeptide is not available for NAD$^+$ formation in a neighboring polypeptide. Thus, in advance of a crystal structure of Qns1, we provide genetic evidence for an obligate intramolecular route for ammonia in Qns1 multimers.

Structural Phylogenetic Excavation of the Qns1 Ammonia Channel—The crystal structure of worm NitFhit (28) and crystal structures of ammonia-dependent NAD$^+$ synthetase from *B. subtilis* (30, 31) were used to construct a low resolution-predictive view of the organization of the putative Qns1 tetramer. Because the Nit tetramer has pairs of C termini directed away from its core structure and bacterial NAD$^+$ synthetase dimers have a pair of N termini that emerge from one specific face, there is a uniquely reasonable way to associate pairs of bacterial NAD synthetase dimers with a Nit tetramer. This organization would create four ~45-Å routes for ammonia within the tetramer, constrained by the genetic data to run from the glutaminase active site to the nicotinic acid β carbon of a NaAD-AMP intermediate *within monomers*. When Qns1 amino acids that are identical in all of the known eukaryotic NAD synthetases were threaded against the predicted structure of Qns1 and *colored red*, 12 residues marked a path between the glutaminase active site and the NAD synthetase active site (Fig. 5). These residues, including Ile-111, Arg-112, Leu-117, Glu-177, Leu-529, Tyr-532, Asp-593, Tyr-601, Leu-604, Met-621, Phe-622, and Leu-625 plus a few additional residues that are identical in all of the known eukaryotic NAD synthetases but not locatable by alignment with Nit or bacterial NAD synthetase, are proposed to protect ammonia from bulk solvent for attack of NaAD-AMP (Fig. 5, *right side*). Thus, beyond the Rosetta Stone fusion event (35) between a nitrilase-related glutaminase and an NAD$^+$ synthetase that created Qns1 homologs, the evolutionary record appears to contain information regarding an ammonia channel that has been found within the best characterized of the GAT-containing enzymes (14).

CONCLUSIONS

Discovery of the glutamine dependence of fungal and human NAD$^+$ synthetases (10, 11) *versus* the ammonia dependence of *E. coli* NAD$^+$ synthetase (12) set up a long standing problem in biochemistry and molecular biology. Not long after the Rosetta Stone hypothesis was advanced as a general method to establish functional relationships between proteins fused in some forms of life (35), the crystal structure of NitFhit was solved (28) and additional domain fusions were discovered within the nitrilase superfamily (16). As part of this analysis, we proposed that a nitrilase-related domain functions as the GAT for eukaryotic NAD$^+$ synthetases and those bacterial NAD$^+$ synthetases that are glutamine-dependent (16). Apparently unaware of these predictions and prior work on the nitrilase superfamily, Tsuchiya and co-workers (33) independently cloned the human Qns1 homolog and demonstrated that glutamine dependence depends on Cys-175. Although they did not explore whether active-site residues are required *in vivo* or whether the enzyme has an obligate intramolecular mechanism for ammonia transfer, they made the surprising discovery of a second NAD$^+$ synthetase that is claimed to be a novel human ammonia-dependent NAD$^+$ synthetase (33). In the accompanying paper, we show that the second enzyme is actually a *pncB*-linked ammonia-dependent NAD$^+$ synthetase from a species of *Pseudomonas* (36).

Although eukaryotic NAD$^+$ synthetases are consistently glutamine-dependent *in vitro*, it has always been possible to measure ammonia-dependent activity *in vitro* as well (13). Here we show that glutamine dependence is necessary for biological function. Biochemical analysis of recombinant Qns1 indicated that the glutaminase active site and the NAD$^+$ synthetase active sites are in communication with each other as glutaminase activity is stimulated ~25-fold by the presence of the NaAD ammonia acceptor. However, mutation of the glutaminase active site did not significantly depress ammonia-dependent NAD$^+$ synthetase activity, and mutation of the NAD$^+$ synthetase active site depressed glutaminase activity approximately to the level of wild-type enzyme assayed in the absence of NaAD. Given the discrete nature of these mutations and the multimeric nature of Qns1, intragenic complementation (34, 37) assays were performed. Biochemical and genetic analysis of the multimeric Qns1 enzyme indicates that ammonia must be channeled from the GAT domain to the NAD$^+$ synthetase domain within Qns1 monomers. Finally, when the sequence of yeast Qns1 was threaded against the determined structure of a Nit tetramer (28) and the determined structure of *B. subtilis* NAD synthetase (31) organized in a manner that would connect the glutaminase and synthetase domains, we discovered that 12 residues conserved in all of the known eukaryotic NAD$^+$ synthetases are found along a 46-Å path between the two active sites. Current work is geared toward illumination of the apparent ammonia channel by mutagenesis and x-ray crystallography.

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