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-ribose)lation. 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 ammoxate 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 glutamin-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 vitro. 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 obligatory 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\(^+\))\(^3\) and its phosphoylated 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. Besides 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 adenyllylated to nicotinic acid dinucleotide (NAD 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.

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

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 deproteinate NH\(_3\), 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...
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 aminocyt-
rNA trans-amidases were described as a third unrelated type of GAT (15). Here we show that a domain related to nitrilase
in vivo
model constructed for the glutaminase and the NAD
that a heteromultimer consisting of polypeptides with muta-
tional GAT and NAD
qns1
merized 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 MATα
MATa
, ura3::kanMX4, his3::LEU2, leu2::HIS3, trp1::HIS3, his3::LEU2, met15::LEU2
and sensitivity to 1 mg/ml 5-fluoroorotic (5FOA). Strain BY165–1d of genotype
GAL1
GAL1
and qns1
were initiated with 270 ng of wild-type or mutant enzymes. Glutamine-dependent
assays were in 0.9 ml of 10 mM sodium pyrophosphate, pH 7.5. After triplicate 60-min, 37 °C incubations, reactions were termi-
nated 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 determina-
tions using either alcohol dehydrogenase or glutamate dehydroge-
rase-coupled assays to determine NAD⁺ and glutamate (26) formation,
respectively. NAD⁺ assays were in 0.9 ml of 10 mM sodium pyrophos-
phate, 0.1% ethanol. Absorbance (340 nm) was measured before and after a 10-min room temperature incubation with 29 units of alcohol
dehydrogenase. NAD⁺ standards (0–100 nM) 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
potassium phosphate, 0.1% ethanol. Absorbance (340 nm) was measured before and after a 2-min centrifugation at 12,000 rpm. For glutamate determinations, glutamate standards (0–32 nM) underwent both incubations in pa-
allel with samples and contained all of the ingredients other than Qns1.

 Immuno precipitation—Wild-type laboratory yeast strain SETY6210

FIG. 1. Reaction scheme of glutamine-dependent NAD⁺ synthetase. Qns1 catalyzes Mg²⁺-dependent ADP-ribosylation of NaAD to form NaAD-AMP. In the second step, glutamine is con-
sumed to glutamate and the released ammonia attacks the adenylated carbon, releasing AMP and forming NAD⁺.
tured proteins were extracted with 8M urea and subjected to SDS-
beads were washed five times with phosphate-buffered saline and cap-
antibody cross-linked to agarose beads. After a 60-min incubation,
dissection. As shown in Fig. 2
marker in a wild-type diploid strain, which was induced to
QNS1
(16),
qns1-E45A QNS1 HIS3
pB379 pRS413
qns1-K114A QNS1 HIS3
pB381 p414GAL1
2xHA-qns1-C175A
pB317 pSGA04
His7-qns1-C175A
pB338 pRS414
His7-qns1-E527A
T7
pB339 pSGA04
His7-qns1-C175A
T7
bla
pB340 pSGA04
His7-qns1-E527A
T7
bla
pB341 p413GAL1
3xFLAG-qns1-E527A
GAL1
His3
pB342 p413GAL1
3xFLAG-qns1-K114A, C175A
GAL1
His3
pB343 p413GAL1
3xFLAG-qns1-E45A, C175A
GAL1
His3
pB358 pSGA04
His7-qns1-E45A
T7
bla
pB359 pSGA04
His7-qns1-K114A
T7
bla
pB378 pRS413
qns1-K114A
T7
pB379 pRS413
qns1-E45A
T7
His3
pB379
(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 cap-
tured proteins were extracted with 8 M urea and subjected to SDS-
PAGE. Duplicate Western blots were performed by probing with
immunohistological conjugate. Immune complexes were detected with chemilumines-
cent peroxidase substrate (Sigma).

RESULTS AND DISCUSSION
QNS1 Is an Essential Yeast Gene—On the basis of its N-
terminal domain of similarity to the nitrate 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. 2A, viability segregated 2 to 2. All
deletion 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. 2B). Transformation of the T. maritima QNS1 plasmid into an isolate with qns1A
covered by S. cerevisiae QNS1 allowed the curing of the yeast
gene, producing isolates that exhibited galactose-dependent
and glucose-repressible growth. Fig. 2C shows this rare case of
complementation of a eukaryotic gene deletion by a correspond-
ing 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. Because 5FOA-
resistant, i.e. Ura-
isolates (27) of strain PB165, could not be

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Table I

| Name        | Vector       | Inert      | Promoter   | Marker    |
|-------------|--------------|------------|------------|-----------|
| pB175       | pRS416       | QNS1       | QNS1       | URA3      |
| pB177       | pRS413       | QNS1       | QNS1       | HIS3      |
| pB178       | pRS413       | qns1-C175A | QNS1       | HIS3      |
| pB229       | p414GAL1     | T. maritima| QNS1       | TRP1      |
| pB251       | pRS413       | qns1-E527A | QNS1       | HIS3      |
| pB252       | pRS413       | qns1-D365A | QNS1       | HIS3      |
| pB254       | p414GAL1     | 2xHA-QNS1  | GAL1       | HIS3      |
| pB254       | p413GAL1     | 2xHA-qns1 | GAL1       | TRP1      |
| pB256       | p414GAL1     | 3xFLAG-qns1 | GAL1   | HIS3      |
| pB256       | p413GAL1     | 2xHA-qns1-E527A | GAL1 | HIS3    |
| pB258       | p414GAL1     | 3xFLAG-qns1-C175A | GAL1 | HIS3    |
| pB258       | p413GAL1     | 3xFLAG-qns1-E527A | GAL1 | HIS3    |
| pB260       | p414GAL1     | 2xHA-qns1-C175A | GAL1 | HIS3    |
| pB260       | p413GAL1     | 3xFLAG-qns1-E527A | GAL1 | HIS3    |

Table II

| Oligodeoxynucleotides used in this study (5’-3’) |
|-----------------------------------------------|
| Name                                           |
| R001                                          |
| R002                                          |
| R003                                          |
| R004                                          |
| 5093                                         |
| 5094                                         |
| 5118                                         |
| 5168                                         |
| 5189                                         |
| 7032                                         |
| 7033                                         |
| 7039                                         |
| 7040                                         |
| 7116                                         |
| 7117                                         |
| 7118                                         |
| 7119                                         |
| 7120                                         |

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P. Bieganowski, H. C. Pace, and C. Brenner, unpublished results.
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 spor 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. 3A), 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 Mg²⁺ ions positioned at the site of adenyllylation/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. 3B, 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 NH₄Cl as the source of ammonia, wild-type Qns1 produced 4% as much NAD⁺ as it did with glutamate 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 glutaminase-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. 3C, 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 *vivo*. 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 phosphoribosylpyrophosphate 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 amido 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⁺
drawn from the heteromultimeric glutamine-dependent NAD synthetase found in nature. Independently, the same conclusion was reached regarding the丢了 the domain is the fourth independently evolved GAT domain in the anticipated reasons. Thus, the nitrilase-related glutaminase active site contains an active site including Asp-365 and Glu-527. 

B. qns1-E527A (pB251), and 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. 

Thermus thermophilus synthetase of NH$_3$-dependent synthetase of human glutamine-dependent NAD synthetase was assayed without NaAD before and after mutation of Cys-175, leading to identification of the nitrilase-related domain as a glutaminase (33).

In vivo establishment 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. 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).

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 tetramer 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. 3. Qns1 contains two essential active sites for NAD$^+$ synthetase function in vivo.](image)

**A**. Domain structure of Qns1. The glutamine amidotransferase domain contains nitrilase-related active-site residues Glu-65, 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. 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 (pB230), qns1-C175A (pB337), 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 the triple mutant allowed colonies to support growth, indicating that residual glutaminase activities of glutaminase active-site mutants are real.
and form a heteromultimer. Presence of glutaminase Qns1-FLAG and anti-HA antibodies (Ab) analyzed by Western blotting 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. 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 fail 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-HA 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 project 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 panel** 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 panel**, 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 nitritase superfamily (16). As part of this analysis, we proposed that a nitritase-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 nitritase 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 vitro 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 pnxB-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 expressed 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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REFERENCES

1. Ziegler, M. (2000) Eur. J. Biochem. 267, 1550–1564
2. Burkle, A. (2001) Bioessays 23, 795–806
3. Rousseau, T. (2000) EMBO J. 19, 1176–1179
4. Grozinger, C. M., and Schreiber, S. L. (2002) Chem. Biol. 9, 3–16
5. Sauer, A. A., Celic, I., Avalos, J., Deng, H., Boeke, J. D., and Schramm, V. L. (2003) Biochemistry 42, 15459–15463
6. Lin, S. J., Defossez, P. A., and Guarente, L. (2000) Science 289, 2126–2128
7. Lin, S. J., Kaeberlein, M., Andalis, A. A., Sturtz, L. A., Defosse, P. A., Culotta, V. C., Fink, G. R., and Guarente, L. (2002) Nature 418, 344–348
8. Bell, S. D., Botting, C. H., Wardsworth, B. N., Jackson, S. P., and White, M. F. (2002) Science 296, 148–151
9. Stari, V. J., Celic, I., Cole, R. N., Boeke, J. D., and Escalante-Semerana, J. C. (2002) Science 298, 2390–2392
10. Press, J., and Handler, P. (1958) J. Biol. Chem. 233, 488–492
11. Press, J., and Handler, P. (1958) J. Biol. Chem. 233, 493–500
12. Spencer, R. L., and Press, J. (1967) J. Biol. Chem. 242, 385–392
13. Yu, C. K., and Dietrich, L. S. (1972) J. Biol. Chem. 247, 4794–4802
14. Zalkin, H., and Smith, J. L. (1998) Adv. Enzymol. Relat. Areas Mol. Biol. 72, 87–144
15. Carnow, A. W., Kw, H., Yuan, R., Si, K. Martina, O., Winkler, W., Henkin, T. M., and Dott, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11819–11826
16. Pace, H. C., and Brenner, C. (2001) Genome Biol. 2, 0011.1–0011.9
17. Cantoni, R., Branzoni, M., Labo, M., Rizzi, M., and Riccardi, G. (1998) J. Bacteriol. 180, 3218–3221
18. Robinson, J. S., Klonsky, D. J., Banta, L. M., and Emr, S. D. (1988) Mol. Cell. Biol. 8, 4596–4594
19. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
20. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
21. Mumberg, D., Muller, R., and Funk, M. (1994) Nucleic Acids Res. 22, 5767–5768
22. Nelson, K. E., Clayton, R. A., Gill, S. R., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D., Nelson, W. C., Ketchum, K. A., McDonald, L., Utterback, T. R., Malek, J. A., Linher, K. D., Garrett, M. M., Stewart, A. M., Cotton, M. D., Pratt, M. S., Phillips, C. A., Richardson, D., Heidelberg, J., Sutton, G. G., Fleischmann, R. D., Eisen, J. A., Fraser, C. M., et al. (1999) Nature 399, 323–329
23. Ghosh, S., and Lowenstein, J. M. (1997) Gene (Amst.) 176, 249–255
24. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Yeast 14, 115–132
25. Kaiser, C., Michaelis, S., and Mitchell, A. (1994) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Riez, T. J., and Davison, T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5177–5186
27. Boeke, J. D., Trueheart, J., Natsoulis, G., and Fink, G. R. (1987) Methods Enzymol. 154, 164–175
28. Pace, H. C., Hodawadekar, S. C., Draganescu, A., Huang, J., Bieganowski, P., Pekarsky, Y., Croce, C. M., and Brenner, C. (2000)Curr. Opin. Biol. 10, 907–917
29. Brenner, C. (2002) Curr. Opin. Struct. Biol. 12, 775–782
30. Rizzi, M., Nesi, C., Mattivi, A., Coda, A., Bolognesi, M., and Galizia, A. (1996) EMBO J. 15, 5125–5134
31. Rizzi, M., Bolognesi, M., and Coda, A. (1998) Structure 6, 1129–1140
32. Bera, A. K., Smith, J. L., and Zalkin, H. (2000) J. Biol. Chem. 275, 7975–7979
33. Harz, N., Yamasu, K., Terasima, M., Osag, H., Shimoyama, M., and Tsuchiya, M. (2003) J. Biol. Chem. 278, 10994–10921
34. Lieb, M. (1976) Mol. Gen. Genet. 146, 291–297
35. Marzocchi, M., Pellegreni, M., Xg. L., Rice, D. W., Yeates, T. O., and Eisenberg, D. (1999) Science 285, 751–753
36. Bieganowski, P., and Brenner, C. (2003) J. Biol. Chem. 278, 33056–33059
37. Yu, B., and Howell, P. L. (2000) Cell. Mol. Life Sci. 57, 1637–1651