

# Glutamine-dependent NAD<sup>+</sup> Synthetase

## HOW A TWO-DOMAIN, THREE-SUBSTRATE ENZYME AVOIDS WASTE\*<sup>§</sup>

Received for publication, July 26, 2006, and in revised form, August 30, 2006 Published, JBC Papers in Press, September 5, 2006, DOI 10.1074/jbc.M60711200

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Glutamine-dependent NAD<sup>+</sup> synthetase, Qns1, utilizes a glutamine aminotransferase domain to supply ammonia for amidation of nicotinic acid adenine dinucleotide (NaAD<sup>+</sup>) to NAD<sup>+</sup>. Earlier characterization of Qns1 suggested that glutamine consumption exceeds NAD<sup>+</sup> production by 40%. To explore whether Qns1 is systematically wasteful or whether additional features account for this behavior, we performed a careful kinetic and molecular genetic analysis. In fact, Qns1 possesses remarkable properties to reduce waste. The glutaminase active site is stimulated by NaAD<sup>+</sup> more than 50-fold such that glutamine is not appreciably consumed in the absence of NaAD<sup>+</sup>. Glutamine consumption exceeds NAD<sup>+</sup> production over the whole range of glutamine and NaAD<sup>+</sup> substrate concentrations with greatest efficiency occurring at saturation of both substrates. Kinetic data coupled with site-directed mutagenesis of amino acids in the predicted ammonia channel indicate that NaAD<sup>+</sup> stimulates the glutaminase active site in the  $k_{cat}$  term by a synergistic mechanism that does not require ammonia utilization by the NaAD<sup>+</sup> substrate. Six distinct classes of Qns1 mutants that fall within the glutaminase domain and the synthetase domain selectively inhibit components of the coordinated reaction.

Glutamine-dependent NAD<sup>+</sup> synthetase Qns1 (1) is one of many enzymes that couples a glutamine amidotransferase (GAT)<sup>2</sup> domain to a second active site that requires ammonia gas as a reactant (2–4). The second active site of Qns1 is an NAD<sup>+</sup> synthetase domain that reacts nicotinic acid adenine dinucleotide (NaAD<sup>+</sup>) with ATP to form an activated, adenylylated intermediate (NaAD-AMP), releasing pyrophosphate. In turn, NaAD-AMP is attacked by ammonia to produce NAD<sup>+</sup> plus AMP. Ideally, the enzyme would produce one equivalent of NAD<sup>+</sup> and AMP for every glutamine hydrolyzed to glutamate. This ideal stoichiometry, depicted in Fig. 1, has been attributed to glutamine-dependent NAD<sup>+</sup> synthetase since the first description of the enzyme by Preiss and Handler (5). The sim-

ilarity of Fig. 1 notwithstanding, characterization of the native (6) and recombinant (1) purified enzyme has never been entirely consistent with ideality. Whether the enzyme is, in fact, capable of perfect stoichiometry, potentially at a “sweet spot” of substrate concentrations, has not been investigated.

Though eukaryotic and some prokaryotic NAD<sup>+</sup> synthetases have long been understood to be glutamine-dependent enzymes (5, 7), the GAT domain of these enzymes initially escaped recognition as a glutaminase because it is neither a triad-type GAT, an N-terminal nucleophile-type GAT (2), nor one related to aminoacyl-tRNA transamidases (8). In the course of characterizing the domain structure of members of the nitrilase superfamily, we observed that the glutamine dependence of all eukaryotic and particular prokaryotic NAD<sup>+</sup> synthetases could be accounted for by the presence of a putative GAT domain related to nitrilase (9, 10). Site-directed mutagenesis of yeast Qns1 coupled to biochemical and physiological experiments established that the nitrilase-related domain functions as a GAT and consists of a Glu-Lys-Cys catalytic triad (1). Experiments indicated that the synthetase and the glutaminase active sites must reside in the same polypeptide for Qns1 to function *in vivo* even though glutaminase mutants retained ammonia-dependent NAD<sup>+</sup> synthetase activity, synthetase mutants retained basal glutaminase activity, and the two mutants assembled into a stable multimer *in vivo* (1). We interpreted these experiments to indicate that there is a specific intramolecular ammonia channel from the GAT active site to the synthetase active site. Modeling and structure-based sequence analysis suggested that the ammonia channel in Qns1 is ~46 Å long and lined with residues conserved among glutamine-dependent NAD<sup>+</sup> synthetases (1).

In the earlier study, we determined that saturating NaAD<sup>+</sup> increases glutaminase activity by at least 30-fold (1). In the present study we have shown that NaAD<sup>+</sup> accelerates the  $k_{cat}/K_m$  of the Qns1 glutaminase active site by ~50-fold with a reversible substrate dissociation constant ( $K_s$ ) that is 24-fold lower than the  $K_m$  of Qns1 for NaAD<sup>+</sup> to NAD<sup>+</sup> product formation. Whereas this mode of regulation reduces the glutamine to NAD<sup>+</sup> efficiency at low NaAD<sup>+</sup> levels, it effectively reduces the waste of glutamine on an absolute basis in the absence of NaAD<sup>+</sup>. Moreover, at high NaAD<sup>+</sup> levels, Qns1 achieves maximal glutamine to NAD<sup>+</sup> efficiency and becomes strictly limited by glutamine levels to produce NAD<sup>+</sup>. The communication between NaAD<sup>+</sup> binding and glutaminase stimulation is reminiscent of carbamoyl phosphate synthetase, which has a

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table S1.

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<sup>2</sup> The abbreviations used are: GAT, glutamine amidotransferase; NaAD<sup>+</sup>, nicotinic acid adenine dinucleotide.

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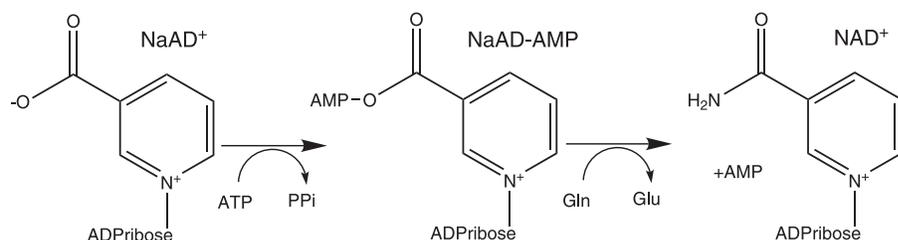


FIGURE 1. **Reaction scheme of glutamine-dependent  $\text{NAD}^+$  synthetase.** According to ideal stoichiometry, Qns1 utilizes one ATP and glutamine per  $\text{NaAD}^+$  substrate, producing one AMP, one inorganic pyrophosphate, one glutamate, one  $\text{NAD}^+$ , and no ammonia.

molecular tunnel for transport of ammonia and carbamate. In the case of carbamoyl phosphate synthetase, glutaminase activity is accelerated 1000-fold by bicarbonate phosphorylation at a site 45 Å away (3, 4, 11).

Precedent for stimulation of one active site by binding a substrate at another active site is found in several classic studies. In analysis of luciferase, McElroy *et al.* (12) termed the phenomenon homosterism, in contradistinction to allosterism, to indicate that a substrate binding event could position a multisubstrate enzyme to react more efficiently with subsequently bound substrates at the normal engagement sites of all substrates. Analyzing succinyl coenzyme A synthetase, Boyer and coworkers (13) made similar observations, which were termed substrate synergism. According, we refer to the ability of  $\text{NaAD}^+$  binding to accelerate the glutaminase active site as homosteric stimulation and as an example of substrate synergism.

To further dissect the efficiency of Qns1 and the communication between the two active sites, we targeted ten amino acids in the predicted ammonia channel of Qns1 for site-directed mutagenesis and characterized purified mutant enzymes physiologically and biochemically, identifying six different classes of mutant enzymes. This set of mutants produced richly distinct phenotypes: glutaminase mutants with poor synthetase activity but retaining good substrate synergism; glutaminase mutants totally dependent on substrate synergism; glutaminase mutants with poor synthetase activity that inhibit substrate synergism; synthetase mutants that depress all activities; synthetase mutants with poor synthetase activity but retaining good substrate synergism; and synthetase mutants with no synthetase activity and no substrate synergism. The combined kinetic and molecular genetic analyses of Qns1 establish a homosteric function for  $\text{NaAD}^+$  binding in the coordination of glutamine consumption with  $\text{NaAD}^+$  availability.

### EXPERIMENTAL PROCEDURES

**Site-directed Mutagenesis**—Plasmid pB337 and derivatives were used for expression of yeast *QNS1* in *Escherichia coli* (1). Site-directed mutagenesis (14) of plasmid pB337 was used to create plasmids to express *QNS1* alleles I111A, R112S, R112L, E177A, D593A, Y601A, L604A, L604N, M621A, and F622A using primers listed in supplemental Table S1. Double mutants (L529A,L604A; Y601A,M621A; Y532A,Y601A; D593A,F622A) were constructed by subjecting particular single mutants to a second round of site-directed mutagenesis. Plasmid pB177, carrying *QNS1* with the native promoter on a single copy *HIS3*

plasmid (1), was the reference for complementation testing of *QNS1* alleles. Mutations were introduced into plasmid pB177 as indicated in supplemental Table S1. All constructs were confirmed by DNA sequencing.

**In Vivo Assays**—Mutant derivatives of plasmid pB177, carrying *QNS1* with the native promoter on a single copy *HIS3* plasmid, were transformed into yeast strain

BY165–1d (genotype *MATa qns1Δ::kanMX4 ura3 trp1 his3 leu2 met15*) carrying pB175, which encodes wild-type *QNS1* on a *URA3* plasmid. Transforming in a functional allele of *QNS1* linked to *HIS3* allowed curing of pB175, scored by 5-fluoroorotic acid resistance. Conversely, nonfunctional alleles were scored by 5-fluoroorotic acid sensitivity (1). On the basis of the 5-fluoroorotic acid plating assay, each allele was scored + or – for functional complementation in Table 1.

**Enzyme Purification**—His-tagged yeast Qns1 proteins were expressed and purified as described previously (1). Protein was measured by the method of Bradford (15), using bovine serum albumin as the standard.

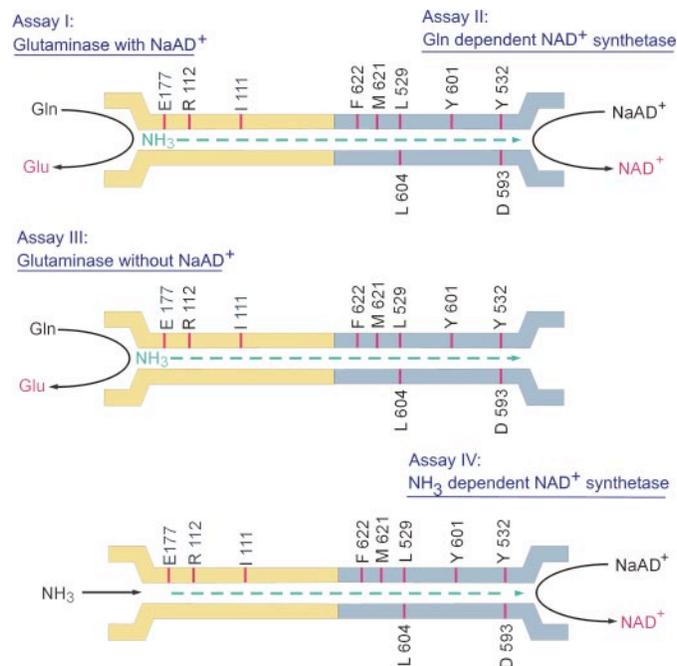
**Enzymatic Assays**—Assays were based on those described earlier (1). Specific activities of Qns1 were determined in reaction buffer (2 mM ATP, 5 mM  $\text{MgCl}_2$ , 50 mM Tris-HCl, pH 8.0, 56 mM KCl, and 0.2 mg/ml bovine serum albumin) with 1 mM  $\text{NaAD}^+$ , 20 mM glutamine, and 0.1  $\mu\text{g}$  of enzyme, incubated for 60 min at 37 °C. Reactions were terminated by heating in a boiling water bath for 3 min, chilled on ice, and centrifuged for 10 min at 12,000 rpm. Supernatants were used for  $\text{NAD}^+$  or glutamate product determination. The assay for the  $\text{NAD}^+$  product contained supernatant, 0.1% ethanol, 10 mM sodium pyrophosphate, and 6.4 units of alcohol dehydrogenase (Sigma) incubated for 60 min at 25 °C.  $\text{NAD}^+$  standards (0–150  $\mu\text{M}$ ) were measured under the same reaction conditions. The glutamate product was determined by incubating reaction supernatant with 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, 0.5 mM  $\text{NAD}^+$ , and 2.4 units of glutamate dehydrogenase (Sigma). Reactions were incubated for 90 min at 37 °C and clarified by a 5-min centrifugation at 12,000 rpm. Glutamate standards (0–70  $\mu\text{M}$ ) underwent both incubations in parallel with assayed samples and contained all of the ingredients other than Qns1. NADH, the final product of both assays, was detected at 340 nm and converted to molar using the extinction coefficient, 6,220  $\text{M}^{-1} \text{cm}^{-1}$ . Specific activity of glutaminase without  $\text{NaAD}^+$  was determined with 20 mM glutamine and 0.9  $\mu\text{g}$  of Qns1. The ammonia-dependent  $\text{NAD}^+$  synthetase assay was performed with 1 mM  $\text{NaAD}^+$  and 2 mM  $\text{NH}_4\text{Cl}$  as an amide donor in the presence of 0.3  $\mu\text{g}$  of Qns1.

Kinetic analyses of the glutaminase activity of Qns1 were completed as described above for specific activity but with varied concentrations of glutamine (0–80 mM) and either a saturating amount of  $\text{NaAD}^+$  (1 mM) or no  $\text{NaAD}^+$ .  $\text{NAD}^+$  synthetase activity of Qns1 was measured by assaying varied concentrations of  $\text{NaAD}^+$  (0–400  $\mu\text{M}$ ) in the presence of saturating glutamine (20 mM). Effects of  $\text{NaAD}^+$  on glutaminase

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and NAD<sup>+</sup> synthetase activities were determined using glutamine concentrations from 0 to 10 mM in NaAD<sup>+</sup> varying from 0 to 600 μM. ATP dependence assays were completed in the same manner but with 0–80 mM glutamine and either 0 or 2 mM ATP in the reaction buffer. Kinetic assays utilized 0.5 μg of

Qns1 and were incubated from 60 to 180 min at 37 °C. ATP and NaAD<sup>+</sup> concentrations were calculated using their extinction coefficient at 259 nm (15,400 M<sup>-1</sup> cm<sup>-1</sup>). In all cases, initial rates were calculated from reactions in which no more than 10% of substrates were converted to products.



**FIGURE 2. Assays utilized for analysis of Qns1 glutaminase and NAD<sup>+</sup> synthetase activities.** Assay I measures glutamate produced by the glutaminase domain in the presence of both glutamine and NaAD<sup>+</sup> and typically ATP. Assay II measures glutamine-dependent NAD<sup>+</sup> formation in the presence of glutamine, NaAD<sup>+</sup>, and ATP. Assay III measures glutaminase activity in the absence of NaAD<sup>+</sup>. Assay IV measures ammonia-dependent NAD<sup>+</sup> formation. Qns1 is depicted as a channel from the glutaminase active site to the synthetase active site. Conserved residues are depicted in their predicted relative locations along the ammonia channel (1) in the nitrilase-related glutaminase domain (yellow) and the synthetase domain (blue).

**TABLE 1**  
Characterization of wild-type and six classes of Qns1 mutants

Enzyme	<i>In vivo</i> function	Specific activity			
		Glutaminase with NaAD <sup>+</sup>	Glutamine-dependent NAD <sup>+</sup> synthetase	Glutaminase without NaAD <sup>+</sup>	NH <sub>3</sub> -dependent NAD <sup>+</sup> synthetase
<i>nmol min<sup>-1</sup> mg<sup>-1</sup></i>					
Wild-type	+	3290 ± 130	2330 ± 70	114 ± 6	741 ± 28
Class I: Glutaminase mutant with poor synthetase activity retaining good substrate synergism					
I111A	+	108 ± 4	53.3 ± 0.3	25.1 ± 0.8	14.3 ± 1.8
Class II: Glutaminase mutant totally dependent on substrate synergism					
E177A	+	127 ± 2	112 ± 1.4	1.1 ± 0.1	631 ± 9
Class III: Glutaminase mutants with poor synthetase activity that inhibit substrate synergism					
R112L	–	1.2 ± 0.4	0.2 ± 0.3	4.2 ± 0.2	0.3 ± 0.1
R112S	–	37.2 ± 1.0	24.0 ± 2.5	17.1 ± 1.3	15.0 ± 1.0
Class IV: Synthetase mutant that depresses all activities					
M621A	+	1130 ± 10	644 ± 3	46.1 ± 1.0	250 ± 2
Class V: Synthetase mutants with poor synthetase activity retaining substrate synergism					
D593A	+	1480 ± 30	435 ± 13	215 ± 3	152 ± 4
L604A	+	531 ± 17	293 ± 12	70.9 ± 4.3	183 ± 5
Y601A	+	257 ± 11	93.1 ± 4.8	82.5 ± 2.9	48.5 ± 3.6
F622A	–	43.3 ± 2.0	28.8 ± 1.2	15.4 ± 0.7	8.0 ± 0.2
L529A,L604A	–	18.3 ± 0.9	15.0 ± 1.0	4.9 ± 0.3	2.7 ± 0.1
L604N	–	50.1 ± 1.4	8.7 ± 0.2	22.5 ± 0.5	4.3 ± 0.1
Class VI: Synthetase dead and no substrate synergism					
Y601A,M621A	–	70.6 ± 1.0	20.2 ± 1.3	66.3 ± 3.2	3.3 ± 0.1
Y532A,Y601A	–	309 ± 8	1.7 ± 0.1	257 ± 5	0 ± 0
D593A,F622A	–	729 ± 10	1.1 ± 0	693 ± 10	1.1 ± 0

## RESULTS

### More Than One Glutamine Is Consumed per NAD<sup>+</sup> Formed

The Qns1 enzyme was presumed to have perfect stoichiometry until proven otherwise. To establish the exact stoichiometry of the Qns1 reaction under a variety of conditions and avoid errors associated with measuring two different products, we wished to convert the glutamate product and the NAD<sup>+</sup> product to a common molecule that could be placed on an absolute scale. The solution to this problem was to link glutaminase assays to production of NADH using nonlimiting amounts of glutamate dehydrogenase and NAD<sup>+</sup> and to link NAD<sup>+</sup> synthetase assays to production of NADH using nonlimiting amounts of ethanol and alcohol dehydrogenase. This experimental solution placed glutamate and NAD<sup>+</sup> assays on a common and absolute scale.

Under conditions of saturating ATP, glutamine, and NaAD<sup>+</sup>, the rate of glutaminase activity was shown to exceed the rate of NAD<sup>+</sup> formation by two different criteria. First, we measured the specific activity of wild-type Qns1 in the four assays schematized in Fig. 2. Second, we determined the initial rates of NAD<sup>+</sup> and glutamate formation to determine the  $k_{cat}$  for NaAD<sup>+</sup> to NAD<sup>+</sup> formation (under conditions of saturating glutamine) and the  $k_{cat}$  for glutamine to glutamate formation (under conditions of saturating NaAD<sup>+</sup>). As shown in Table 1, in specific activity terms and under condition of near saturation of both active sites, wild-type Qns1 consumes 3300 nmol/min/mg glutamine while producing only 2300 nmol/min/mg of NAD<sup>+</sup>. This represents a 40% excess of glutamine to NAD. In

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**TABLE 2**  
Kinetic parameters for wild-type and mutant Qns1

Assay	Parameter	Wild type	Y601A	Y532A,Y601A
NaAD <sup>+</sup> → NAD <sup>+</sup> at 20 mM Gln	$k_{cat}$ (s <sup>-1</sup> )	5.4 ± 0.3	0.09 ± 0.02	0.0024 ± 0.0001
	$K_m$ (mM)	0.15 ± 0.01	0.10 ± 0.02	2.8 ± 0.2
	$k_{cat}/K_m$ (s <sup>-1</sup> M <sup>-1</sup> )	36,000	900	0.86
Glutaminase at 1 mM NaAD <sup>+</sup>	$k_{cat}$ (s <sup>-1</sup> )	6.4 ± 0.3	0.16 ± 0.02	0.12 ± 0.01
	$K_m$ (mM)	5.3 ± 2.0	1.4 ± 0.2	3.5 ± 0.1
	$k_{cat}/K_m$ (s <sup>-1</sup> M <sup>-1</sup> )	1200	110	34
Glutaminase without NaAD <sup>+</sup>	$K_{cat}$ (s <sup>-1</sup> )	0.12 ± 0.03	0.03 ± 0.01	0.08 ± 0.01
	$K_m$ (M)	2.9 ± 0.8	1.6 ± 0.3	2.5 ± 0.2
	$k_{cat}/K_m$ (s <sup>-1</sup> M <sup>-1</sup> )	41	19	32

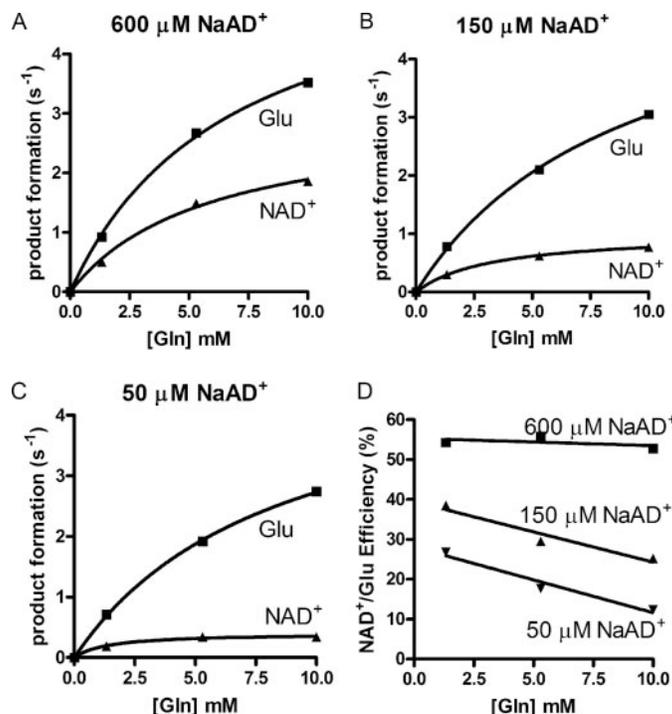
$k_{cat}$  terms, as shown in Table 2, wild-type Qns1 turns over 6.4 glutamine molecules/s/active site when provided with 1 mM NaAD<sup>+</sup> (~7-fold over the 0.15 mM  $K_m$  of NaAD<sup>+</sup> for NAD<sup>+</sup> formation). However, wild-type Qns1 produces only 5.4 NAD<sup>+</sup> products/s/active site when provided with 20 mM glutamine (~4-fold over the 5.3 mM  $K_m$  of Gln for Glu formation). The  $k_{cat}$  comparison, representing the conceivable maximal rates of each active site, still indicates that Qns1 wastes a 20% excess of glutamine in production of NAD<sup>+</sup> when both active sites are saturated.

### Glutaminase $k_{cat}$ Is Accelerated by NaAD<sup>+</sup>

In an earlier study, we showed that the E45A, K114A, and C175A substitutions in the nitrilase-related glutaminase domain and the D365A and E527A substitutions in the NAD<sup>+</sup> synthetase domain inactivate Qns1 function *in vivo* (1). Moreover, although all of these mutants severely inhibited the formation of NAD<sup>+</sup> from glutamine and NaAD<sup>+</sup>, the glutaminase domain mutants did not inhibit formation of NAD<sup>+</sup> from ammonia and NaAD<sup>+</sup>, and the synthetase domain mutants did not inhibit the basal glutaminase activity (1). As further illustrated in Table 1, wild-type Qns1 exhibits a nearly 30-fold increase in glutaminase-specific activity when provided with 1 mM NaAD<sup>+</sup>. Because the E527A active site mutant did not accelerate glutaminase activity in the presence of NaAD<sup>+</sup> (1), we wished to examine the kinetic and physical basis for stimulation of glutaminase activity by NaAD<sup>+</sup>. As seen in Table 2, NaAD<sup>+</sup> increases glutaminase activity >50-fold in the  $k_{cat}$  term, from 0.12 to 6.4 s<sup>-1</sup> with little effect on the  $K_m$  of Gln. Thus, the NaAD<sup>+</sup>-bound form of Qns1 has a higher glutaminase activity rather than a higher affinity for glutamine binding.

### The $K_s$ -NaAD<sup>+</sup> for Glutaminase Stimulation Is Lower than the $K_m$ -NaAD<sup>+</sup> for NAD<sup>+</sup> Formation

A simple hypothesis to explain the synthetase active site-dependent (1) acceleration of glutaminase  $k_{cat}$  requires invoking a pseudo-active site or reactant role for NaAD<sup>+</sup> "pulling" the ammonia product from the glutamine substrate. According to this view, the NaAD-AMP intermediate produced by NaAD<sup>+</sup> binding to the synthetase active site might catalyze glutaminase activity by functioning as an ammonia acceptor. Failure of the synthetase-dead E527A mutant to accelerate glutaminase activity in the presence of NaAD<sup>+</sup> (1) is consistent with this view. Moreover, we reasoned that glutamine-dependent NAD<sup>+</sup> synthetase might be tuned to function with maximal efficiency at some ideal concentration of substrates. For example, if the glutaminase  $k_{cat}$  is 20% too high for the synthetase  $k_{cat}$  then,



**FIGURE 3. Effect of NaAD<sup>+</sup> concentration on reaction efficiency.** Rate curves for glutaminase (■) and NAD<sup>+</sup> synthetase (▲) at different concentrations of NaAD<sup>+</sup>. A, 600 μM NaAD<sup>+</sup>. B, 150 μM NaAD<sup>+</sup>. C, 50 μM NaAD<sup>+</sup>. D, a plot of NAD<sup>+</sup> per glutamine efficiency as a function of concentrations of NaAD<sup>+</sup> and glutamine substrates.

potentially, the enzyme might have evolved to function lower on the glutamine rate curve than on the NaAD<sup>+</sup> rate curve in order to match the active sites.

As shown in Fig. 3, we titrated NaAD<sup>+</sup> above and below the 150 μM  $K_m$  for NaAD<sup>+</sup> to NAD<sup>+</sup> formation and measured both NAD<sup>+</sup> formation and glutaminase activity as a function of glutamine concentration. At 600 μM NaAD<sup>+</sup> (4 times the  $K_m$ -NaAD<sup>+</sup> for NAD<sup>+</sup> formation), the enzyme produces 0.6 equivalents of NAD<sup>+</sup> for every glutamine consumed over the whole range of glutamine concentrations. In this manner, when NaAD<sup>+</sup> is saturating, NAD<sup>+</sup> production is limited simply by glutamine availability and efficiency cannot be improved by dialing down the glutamine concentration (Fig. 3, A and D).

In contrast to the simple behavior at saturating NaAD<sup>+</sup>, the enzyme exhibited strikingly wasteful behavior at lower concentrations of NaAD<sup>+</sup>. For example, at 150 μM NaAD<sup>+</sup> (Fig. 3B), at which the enzyme is half-maximal for NAD<sup>+</sup> production with saturating glutamine, and at 50 μM NaAD<sup>+</sup> (Fig. 3C), at which the enzyme has a very poor rate of NAD<sup>+</sup> formation at saturat-

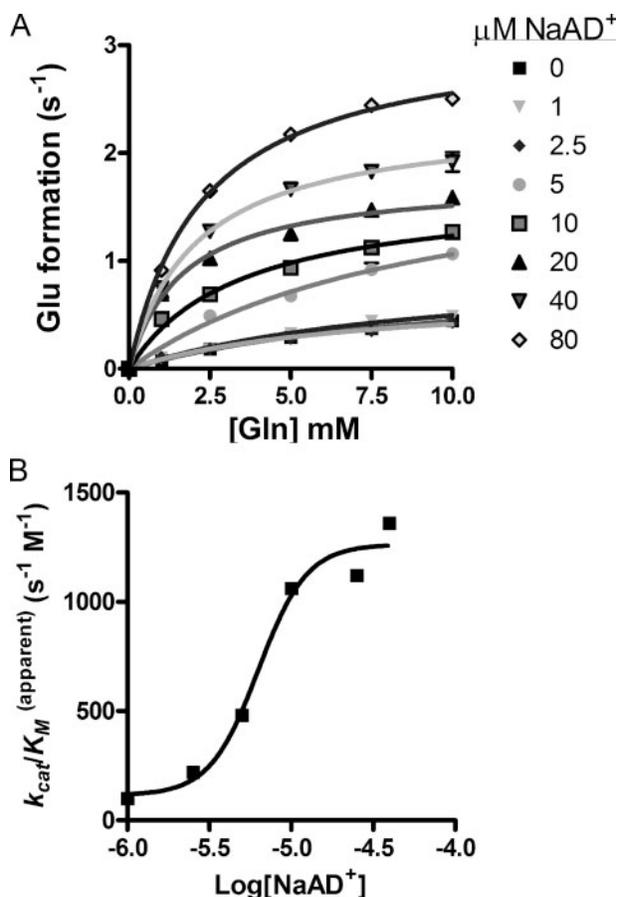


FIGURE 4. NaAD<sup>+</sup> stimulation of the glutaminase active site occurs well below the  $K_m$ -NaAD<sup>+</sup> for NAD<sup>+</sup> formation. Glutaminase activity was measured without NaAD<sup>+</sup> and at seven concentrations of NaAD<sup>+</sup> from 1 to 80 mM. Initial rate data are plotted in panel A. To calculate a  $K_s$ -NaAD<sup>+</sup> for stimulation of the glutaminase active site, the  $k_{cat}/K_m^{(apparent)}$  of the glutaminase activity was determined at each concentration of NaAD<sup>+</sup> and plotted against NaAD<sup>+</sup> concentration. As shown in panel B,  $K_s$ -NaAD<sup>+</sup> for stimulation of the glutaminase active site (midpoint of the sigmoidal activation plot) is at 6.3 mM, 24-fold lower than the  $K_m$ -NaAD<sup>+</sup> for NAD<sup>+</sup> formation.

ing glutamine, the glutaminase was greatly stimulated in excess of NAD<sup>+</sup> production. The poorest efficiencies measured for NAD<sup>+</sup> production per glutamine are at high glutamine and low NaAD<sup>+</sup> (Fig. 3D). Thus, at 50  $\mu$ M NaAD<sup>+</sup> and 10 mM glutamine, the synthetase active site is so slow with respect to the glutaminase active site that 88% of the glutamine is wasted.

The rate curves in Fig. 3 clearly indicate that ammonia consumption by the NaAD<sup>+</sup> reactant is not required for NaAD<sup>+</sup> binding to stimulate glutaminase activity. To calculate the reversible substrate dissociation constant  $K_s$  for the ability of NaAD<sup>+</sup> to stimulate the rate of glutaminase from the basal rate of 0.12 s<sup>-1</sup> to the fully accelerated rate of 6.4 s<sup>-1</sup>, we assayed glutaminase activity in the presence of 0–80  $\mu$ M NaAD<sup>+</sup> over a range of glutamine concentrations (1–10 mM) (Fig. 4A). We determined the  $k_{cat}$  and  $K_m$  values at each NaAD<sup>+</sup> concentration and plotted the glutaminase  $k_{cat}/K_m^{(apparent)}$  as a function of the log of NaAD<sup>+</sup> concentration. As shown in Fig. 4B, half-maximal stimulation of glutaminase in  $k_{cat}/K_m^{(apparent)}$  terms occurs at 6.3  $\mu$ M NaAD<sup>+</sup>. Therefore, NaAD<sup>+</sup> binding to Qns1 with a  $K_s$  of 6.3  $\mu$ M converts it from the glutaminase slow form

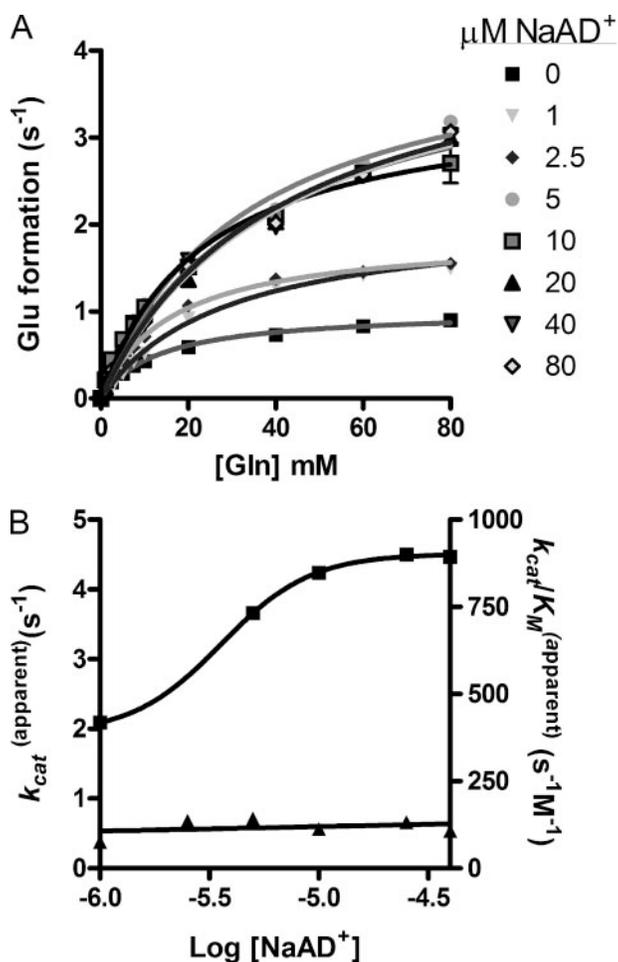


FIGURE 5. NaAD<sup>+</sup> stimulation of the glutaminase active site without ATP is offset by elevated  $K_m$ -Gln. Glutaminase activity was measured without NaAD<sup>+</sup> and at seven concentrations of NaAD<sup>+</sup> from 1 to 80 mM in assays performed without ATP. Initial rate data are plotted in panel A. In panel B, the NaAD<sup>+</sup> concentration dependence for stimulation of  $k_{cat}^{(apparent)}$  (■) and  $k_{cat}/K_m^{(apparent)}$  (▲) of the glutaminase active site are plotted.

to the glutaminase fast form. Remarkably, the  $K_s$ -NaAD<sup>+</sup> is 24-fold lower than the  $K_m$  of NaAD<sup>+</sup> for NAD<sup>+</sup> formation. This indicates that NaAD<sup>+</sup> is bound by Qns1 in a manner that does not result in ammonia consumption by the NaAD-AMP reactant that, nonetheless, stimulates glutaminase activity. Moreover, this unanticipated homosteric regulation of the Qns1 glutaminase active site suggests that Qns1 evolved to function with highest efficiency at high levels of the NaAD<sup>+</sup> substrate.

#### Stimulation of the Glutaminase Active Site by NaAD<sup>+</sup> Depends, in Part, on ATP

The fact that NaAD<sup>+</sup> binding to Qns1 accelerates glutamine consumption well below the  $K_m$  for NAD<sup>+</sup> formation suggested the possibility that NaAD<sup>+</sup> binding without the formation of an NaAD-AMP intermediate might stimulate glutaminase. We tested this hypothesis by titrating NaAD<sup>+</sup> and glutamine in reactions not including ATP. Inspection of the rate curves in Fig. 5A revealed that NaAD<sup>+</sup> in the absence of ATP binding increases both the  $K_m$  and  $k_{cat}$  of the glutaminase. As shown in Fig. 5B, although there is a  $k_{cat}$  stimulation of

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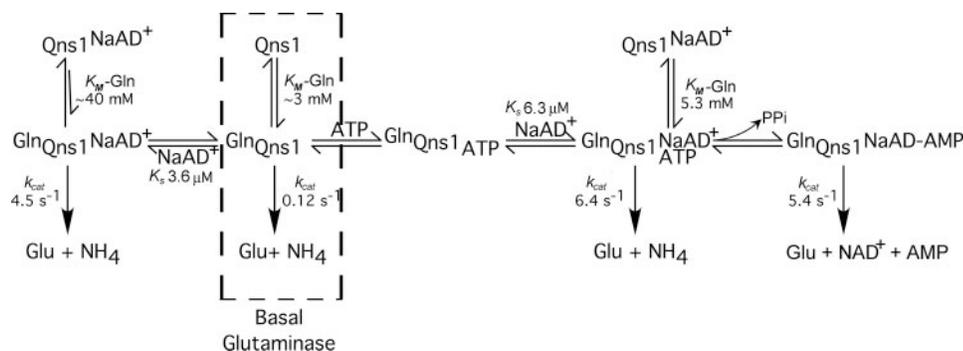


FIGURE 6. **A kinetic model for Qns1 function.** The basal glutaminase reaction is indicated in the *dashed box*. To the *left*, binding of  $\text{NaAD}^+$  is depicted without ATP binding. This form of the enzyme has an accelerated  $k_{\text{cat-Gln}}$  at the expense of increased  $K_{\text{m-Gln}}$ . To the *right*, binding of  $\text{NaAD}^+$  is depicted in the presence of ATP. This form of the enzyme, achieved half-maximally at  $6.3 \mu\text{M}$   $\text{NaAD}^+$ , is 50-fold stimulated in  $k_{\text{cat-Gln}}$  even though the  $K_{\text{m-NaAD}^+}$  for  $\text{NAD}^+$  formation is  $150 \mu\text{M}$ . As explained in the text, the quaternary complex of Qns1 bound to Gln,  $\text{NaAD}^+$ , and ATP can either turn over Gln, forming Glu plus ammonia, or form the catalytically competent Qns1-Gln- $\text{NaAD-AMP}$  intermediate, which forms Glu,  $\text{NAD}^+$ , and AMP.

glutaminase by  $\text{NaAD}^+$  in the absence of ATP, the  $k_{\text{cat}}$  stimulation is offset by impaired glutaminase- $K_{\text{m}}$  such that the  $k_{\text{cat}}/K_{\text{m}}$  (apparent) is static over the whole range of  $\text{NaAD}^+$  concentrations. The results indicate that Qns1- $\text{NaAD}^+$  binds glutamine poorly, although Qns1- $\text{NaAD}^+$ -Gln is a faster glutaminase than Qns1-Gln. The  $K_{\text{s-NaAD}^+}$  for stimulation of  $k_{\text{cat}}$  glutaminase is  $3.6 \mu\text{M}$ .

### A Kinetic Model for Qns1 Function

A kinetic model for Qns1 function is depicted in Fig. 6. Qns1 has a basal glutaminase activity ( $k_{\text{cat}} = 0.12 \text{ s}^{-1}$ ,  $K_{\text{m}} = 3 \text{ mM}$ ) that occurs in the absence of the  $\text{NAD}^+$  synthetase domain substrates ATP and  $\text{NaAD}^+$ . In the absence of ATP,  $\text{NaAD}^+$  ( $K_{\text{s}} = 3.6 \mu\text{M}$ ) can bind Qns1-Gln and one can measure an accelerated  $k_{\text{cat}}$  glutaminase. However, examination of the  $\text{NaAD}^+$ -stimulated glutaminase values without ATP ( $k_{\text{cat}} = 4.5 \text{ s}^{-1}$ ,  $K_{\text{m}} = 40 \text{ mM}$ ) reveals that the  $K_{\text{m-Gln}}$  is greater than twice the concentration of glutamine observed *in vivo* (16). Thus, it would be reasonable to say that binding of  $\text{NaAD}^+$  to Qns1-Gln in the absence of ATP causes Gln to dissociate. In contrast, in the presence of ATP and  $\text{NaAD}^+$  ( $K_{\text{s}} = 6.3 \mu\text{M}$ ), the glutaminase domain is catalytically accelerated  $\sim 50$ -fold with little alteration of the basal  $K_{\text{m-Gln}}$ . Although  $6.3 \mu\text{M}$   $\text{NaAD}^+$  in the presence of ATP converts Qns1 from a slow to a fast glutaminase, this form of the enzyme is largely incompetent to accept ammonia and produce  $\text{NAD}^+$ . Thus, we hypothesize that the glutaminase is conformationally stimulated by binding to ATP and  $\text{NaAD}^+$  substrates without forming a catalytically competent  $\text{NaAD-AMP}$  reactant. The quaternary complex Qns1-Gln- $\text{NaAD}^+$ -ATP can either wastefully turn over and rebound Gln or it can form the adenylylated intermediate, Qns1-Gln- $\text{NaAD-AMP}$ . Driving the reaction to maximal efficiency at saturating levels of all substrates, Qns1 produces 6.4 Glu and 5.4  $\text{NAD}^+$  (and a calculated 1  $\text{NH}_4$ ) from the quaternary complex per second. This suggests that the rate of formation of the adenylylated intermediate is favored over stimulated glutaminase activity such that most, but not all, of the ammonia gas produced by glutaminase activity can be harnessed for  $\text{NAD}^+$  formation.

### Six Classes of Mutants in the Qns1 Ammonia Channel Alter Coordination of the Two Active Sites

The observation that  $\text{NaAD}^+$  has a homosteric function in stimulating glutaminase activity that is kinetically separable from  $\text{NAD}^+$  formation prompted us to investigate the function of ten amino acids that are phylogenetically conserved in eukaryotic  $\text{NAD}^+$  synthetases and physically located in a path from the predicted site of glutamine hydrolysis in the GAT domain to the site of ammonia utilization in the synthetase domain (1). Ten single and four double mutants along

the predicted ammonia channel were constructed in Qns1. Each enzyme was purified to homogeneity and characterized by the four assays depicted in Fig. 1. In addition, each mutant was subject to an *in vivo* complementation test. Based on specific activity characterization presented in Table 1, we divided the fourteen mutants into six classes.

**Class I: Glutaminase Mutant with Poor Synthetase Activity Retaining Good Substrate Synergism**—The first class of Qns1 mutants is represented by the I111A substitution, in which a butyl group in the putative ammonia channel within the glutaminase domain was trimmed back by mutagenesis to a methyl group. Despite the fact that the substitution falls in the glutaminase domain, the most severe defect in the mutant is a 50-fold depression of  $\text{NAD}^+$  synthetase activity with either  $\text{NH}_4\text{Cl}$  or glutamine. Mapping a defective ammonia utilization activity, even for ammonia-dependent  $\text{NAD}^+$  synthetase activity, to the glutaminase domain suggests that ammonia may traverse both domains as a desolvated gaseous reactant as depicted in Fig. 2. The basal glutaminase activity of this mutant is depressed more than 4-fold with respect to wild-type basal glutaminase activity and the mutant retains a greater than 4-fold stimulation of glutaminase by  $\text{NaAD}^+$ , though this is lower than the  $\sim 30$ -fold stimulation of wild-type glutaminase by  $\text{NaAD}^+$  (Table 1). Thus, the Ile side chain apparently contributes greatly to ammonia utilization and significantly to glutaminase activity and substrate synergism. In addition, this depressed mutant is wasteful, converting only half of the glutamine consumed to  $\text{NAD}^+$  produced.

**Class II: Glutaminase Mutant Totally Dependent on Substrate Synergism**—In contrast to the primary defect in synthetase activity of the class I glutaminase mutant, the E177A substitution, which maps to a conserved acidic group closer to the glutaminase active site (Fig. 2), primarily affects the glutaminase active site. Termed class II, this mutant depresses the basal glutaminase activity by more than 100-fold while leaving the ammonia-dependent  $\text{NAD}^+$  synthetase activity nearly unaltered (Table 1). Remarkably, however, the glutaminase activity is restored more than 100-fold by  $\text{NaAD}^+$ , such that this enzyme functions as a highly efficient  $\text{NAD}^+$  synthetase per

glutamine consumed, though the overall activity is depressed 20-fold with respect to wild type. The Glu residue is not essential for *in vivo* activity as this glutaminase-depressed but synergism-retaining mutant was able to support growth in the absence of wild-type enzyme (Table 1). This class II mutant illustrates that efficient basal glutaminase activity is separable from homosteric stimulation of the glutaminase by NaAD<sup>+</sup>.

**Class III: Glutaminase Mutants with Poor Synthetase Activity That Inhibit Substrate Synergism**—The third class of mutants contains enzymes with two substitutions for the conserved guanidino group Arg-112, which is between Glu-177 and Ile-111 along the putative ammonia channel. These mutants produced a lethal phenotype. Biochemical analysis of purified R112S and R112L enzymes indicated that the conserved Arg in the glutaminase domain is required for NAD<sup>+</sup> synthetase activity, with the R112S mutant depressing both synthetase assays 50–100-fold and the R112L mutant depressing the synthetase activities 2,000–10,000-fold (Table 1). However, class III mutants were not just quantitatively more defective than class I. The Arg-112 substituted proteins were also depressed in basal glutaminase activity and did not respond to NaAD<sup>+</sup> either in production of glutamate or NAD<sup>+</sup>. Although earlier we reported loss of function mutants in Qns1 by targeting each of the conserved active site residues in the glutaminase domain (Glu-45, Lys-114, and Cys-175), these residues did not depress ammonia-dependent NAD<sup>+</sup> synthetase activity but rather eliminated Qns1 function specifically by eliminating glutamine-dependent NAD<sup>+</sup> synthetase activity (1). Thus, the class III mutants represent a novel essential function of the glutaminase domain.

**Class IV: Synthetase Mutant that Depresses All Activities**—The class IV mutant, consisting of a M621A substitution in the NAD<sup>+</sup> synthetase domain, reduced specific activity in all four assays by ~3-fold (Table 1). Because both basal and NaAD<sup>+</sup>-stimulated glutaminase activities are decreased by these synthetase domain mutants, class IV mutants influence the communication between the glutaminase domain and the synthetase domain. Therefore, class I and class IV mutants, both of which fall along the putative ammonia channel but affect the opposite active site, reinforce the same theme.

**Class V: Synthetase Mutants with Poor Synthetase Activity Retaining Substrate Synergism**—The class V mutants have modifications in the synthetase domain that result in poor synthetase activity but retain homosteric NaAD<sup>+</sup> acceleration of the glutaminase domain. The basal level of glutaminase activity in these mutants is comparable with wild type (2-fold greater to 7-fold less), with the exception of the double mutant (L529A,L604A), which shows a 23-fold decrease in this activity. Addition of NaAD<sup>+</sup> accelerates the glutaminase activity of all of these mutants 2- to 7-fold but not to the degree observed for wild type (30-fold in specific activity terms). The D593A mutation, which corresponds to an Asp involved in NaAD<sup>+</sup> binding in both *Bacillus subtilis* and *E. coli* NAD<sup>+</sup> synthetases (Asp-220 and Asp-223, respectively) (17, 18), results in an increase of the basal glutaminase activity. Thus, this charge-neutralizing substitution in *Saccharomyces cerevisiae* Qns1 may cause structural adjustments similar to those induced by NaAD<sup>+</sup> binding. The L604A and L604N substitutions suggest a salutary contribution of a nonpolar amino acid at position 604 because the Asn

substitution resulted in a greater loss of enzyme activity in all four assays (Table 1). The Y601A substitution shows decreased NAD<sup>+</sup> synthetase activity with both glutamine and ammonia, although the basal glutaminase activity is only slightly lower than that of wild type. Thus, the glutaminase domain is functional and is stimulated by NaAD<sup>+</sup>, resulting in a more wasteful ratio of glutamine consumed to NAD<sup>+</sup> formed. The L529A,L604A double mutant has even lower NAD<sup>+</sup> synthetase activity with ammonia (68-fold) and glutamine (29-fold) than the other class V mutants. Additionally, this mutant has reduced basal and NaAD<sup>+</sup>-stimulated glutaminase activity but still shows some stimulation by NaAD<sup>+</sup> as is the case with the F622A-substituted enzyme.

**Class VI: Synthetase Dead and No Substrate Synergism**—The class VI mutants have basal glutaminase activities that are similar to or higher than those of wild type. These mutants have virtually no ammonia-dependent NAD<sup>+</sup> synthetase activity, are hundreds- to thousands-fold depressed in glutamine-dependent NAD<sup>+</sup> synthetase activity, and show no stimulation in glutaminase by NaAD<sup>+</sup>. These mutants were obtained by combining the Y601A Class V mutant with additional substitutions in the synthetase domain.

#### *In Vivo* and Kinetic Analysis of Qns1 Mutants

Each mutant allele of the *QNS1* gene was cloned under the control of the *QNS1* promoter on a *HIS3* plasmid that could be propagated at single copy in yeast (1). By transforming these plasmids into a yeast strain carrying *qns1* and *ura3* deletions covered by a plasmid encoding wild-type *QNS1* and the *URA3* gene, we evaluated the ability of each mutant *qns1* gene to support yeast cell growth on the basis of a 5-fluoroorotic acid selection for loss of the *URA3*-linked wild-type *QNS1* plasmid. In assays of glutamine-dependent NAD<sup>+</sup> synthetase, the two lowest specific activity mutants to support the function of yeast cells were the Class I mutant Qns1-I111A and the Class V mutant Qns1-Y601A. Although these mutants possessed only 2.3–4.0% of wild-type specific activity, the viability of each mutant was interesting in light of comparisons with inviable mutants. All eight mutants possessing less than 1.3% of wild-type glutamine-dependent NAD<sup>+</sup> synthetase-specific activity, including both Class III mutants, the three most debilitated Class V mutants, and the three Class VI mutants, were inviable *in vivo*.

In the case of the viable I111A mutant, the ammonia-dependent NAD<sup>+</sup> synthetase activity and basal glutaminase activities were almost identical to those of the inviable Class III mutant, R112S. However, whereas the Class I mutant retained the ability to have glutaminase activity stimulated by NaAD<sup>+</sup> binding, the inviable Arg-112 substitutions in the glutaminase domain were refractory to NaAD<sup>+</sup>, either in production of glutamate or NAD<sup>+</sup>.

In specific activity terms, glutamine-dependent NAD<sup>+</sup> synthetase activity was depressed 25-fold by the Y601A substitution, allowing viability, but addition of the M621A or the Y532A substitutions reduced allosteric activation of the glutaminase by NaAD<sup>+</sup> binding, reduced NAD<sup>+</sup> synthetase activities, and eliminated *in vivo* function. Indeed, we had predicted that the Y532A alteration, which was mapped to the synthetase end of the putative ammonia channel by modeling (Fig. 2) (1), and

## Substrate Coordination and Synergism of NAD<sup>+</sup> Synthetase

which corresponds to the nicotinic acid-binding Phe-170 of *E. coli* NAD<sup>+</sup> synthetase (18), might eliminate the ability of glutamine-dependent NAD<sup>+</sup> synthetase to respond to the NaAD<sup>+</sup> substrate.

To determine how specific activity measurements compare with initial rate measurements and to understand the basis for the lethal defect in Y532A,Y601A, we performed a kinetic analysis of the Y601A single and Y532A,Y601A double mutants. The Y601A mutant, which was characterized in specific activity assays at 4, 72, and 8% of wild-type glutamine-dependent NAD<sup>+</sup> synthetase activity, basal, and NaAD<sup>+</sup>-stimulated glutaminase activities (Table 1), was characterized in  $k_{\text{cat}}/K_m$  terms at 2.5, 46, and 9% of wild-type values (Table 2). However, as shown in kinetic analysis of the Y532A,Y601A double mutant in Table 2, the second alteration in the synthetase active site renders the mutant near normal in basal glutaminase activity but refractory to homosteric stimulation of glutaminase activity by NaAD<sup>+</sup>. Glutamine-dependent NAD<sup>+</sup> synthetase activity, which was reduced >1000-fold in specific activity assays, was reduced 2000-fold in the  $k_{\text{cat}}$  term and increased almost 20-fold in the  $K_m$ -NaAD<sup>+</sup> term. Earlier we showed that the wild-type enzyme accelerates  $k_{\text{cat}}$ -Gln 50-fold with addition of NaAD<sup>+</sup> and ATP and that NaAD<sup>+</sup> addition without ATP accelerates  $k_{\text{cat}}$ -Gln at the cost of elevated  $K_m$ -Gln. Loss of the glutaminase stimulation activity concurrent with loss of NaAD<sup>+</sup> utilization by the Y532A,Y601 mutant is consistent with the dual role of the synthetase domain in accelerating glutaminase and forming NAD<sup>+</sup>.

### DISCUSSION

Our analysis establishes two roles for the NaAD<sup>+</sup> substrate in regulation of glutamine-dependent NAD<sup>+</sup> synthetase. First, with a reversible substrate dissociation constant,  $K_s$ , of 6.3  $\mu\text{M}$ , binding of NaAD<sup>+</sup> converts Qns1 from a slow (0.12 s<sup>-1</sup>) to a fast (6.4 s<sup>-1</sup>) glutaminase without significantly altering the  $K_m$ -Gln as long as ATP is also bound. Despite the ATP requirement for acceleration of glutaminase  $k_{\text{cat}}/K_m$ , the bound NaAD<sup>+</sup> does not appear to be catalytically competent for ammonia consumption as would be expected for an NaAD-AMP intermediate because, as shown in Figs. 3 and 4, Qns1 is very wasteful of glutamine at intermediate concentrations of NaAD<sup>+</sup> and the  $K_m$ -NaAD<sup>+</sup> for NAD<sup>+</sup> formation is not achieved until 150  $\mu\text{M}$ . This implies, as depicted in Fig. 6, that conversion of the quaternary complex, Qns1-Gln-NaAD<sup>+</sup>-ATP, to Qns1-Gln-NaAD-AMP is a nearly rate-limiting step that competes with glutamine turnover. Pushed to maximal rates and efficiency by saturation of all substrates, the glutaminase (6.4 s<sup>-1</sup>) exceeds the synthetase (5.4 s<sup>-1</sup>) by 1 turnover/s, due to partition of the quaternary complex between stimulated glutaminase activity and NaAD-AMP intermediate formation. According to our kinetic scheme (Fig. 6), which is substantially more detailed than the simple stoichiometric model (Fig. 1), the Qns1-Gln-NaAD-AMP intermediate does not waste glutamine; glutamine is only wasted en route to this intermediate.

As shown in Fig. 3D, at saturated NaAD<sup>+</sup> and AMP substrates, the enzyme minimizes glutamine waste and the rate of

forming NAD<sup>+</sup> is limited by glutamine availability. Thus, it is not possible to "dial down" glutaminase activity to match the consumption of NaAD<sup>+</sup>. Instead, a constant percentage of the quaternary complex turns over glutamine rather than going forward to intermediate formation.

Much of the catalytic power of a GAT domain involves protection of ammonia gas as a reactant (2–4, 11). In this study, we identified two classes (I and III, involving amino acid substitutions at amino acids 111 and 112) of mutants in the glutaminase domain that inhibit synthetase activity, using either glutamine or ammonia as the amide donor. These data suggest that, even for ammonia-dependent NAD<sup>+</sup> synthesis, the amide donor is delivered through a specific channel and not bulk solvent.

Mutants along the predicted ammonia channel in either domain were capable of depressing activity in both active sites, one active site, and/or altering communication between the active sites. The enzyme seems to have reduced waste of glutamine by accelerating glutaminase 50-fold by engagement of NaAD<sup>+</sup> and ATP. The cost of this arrangement is formation of a quaternary complex that is competent to produce glutamate or form the NaAD-AMP intermediate. Whether the cell has a way to measure excess glutamine consumption to up-regulate production of NaAD<sup>+</sup> or to accelerate the formation of the NaAD-AMP intermediate *in vivo* are matters for future investigation.

*Acknowledgment*—We thank an anonymous reviewer who directed us to the work of Paul Boyer describing substrate synergism.

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