Secretion of Quinolinic Acid, an Intermediate in the Kynurenine Pathway, for Utilization in NAD<sup>+</sup> Biosynthesis in the Yeast Saccharomyces cerevisiae

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NAD<sup>+</sup> is synthesized from tryptophan either via the kynurenine (de novo) pathway or via the salvage pathway by reutilizing intermediates such as nicotinic acid or nicotinamide ribose. Quinolinic acid is an intermediate in the kynurenine pathway. We have discovered that the budding yeast Saccharomyces cerevisiae secretes quinolinic acid into the medium and also utilizes extracellular quinolinic acid as a novel NAD<sup>+</sup> precursor. We provide evidence that extracellular quinolinic acid enters the cell via Tna1, a high-affinity nicotinic acid permease, and thereby helps to increase the intracellular concentration of NAD<sup>+</sup>. Transcription of genes involved in the kynurenine pathway and Tna1 was increased, responding to a low intracellular NAD<sup>+</sup> concentration, in cells bearing mutations of these genes; this transcriptional induction was suppressed by supplementation with quinolinic acid or nicotinic acid. Our data thus shed new light on the significance of quinolinic acid, which had previously been recognized only as an intermediate in the kynurenine pathway.

MATERIALS AND METHODS

Strains and plasmids. Standard media, such as synthetic complete (SC) and yeast extract-yeast-peptone-dextrose (YPD) media, were used for the cultivation of S. cerevisiae (20). S. cerevisiae was grown aerobically at 30°C in liquid medium. To examine the growth phenotype on solid medium, diluted cell suspensions (4.0 μl; A<sub>600</sub> of 2, 0.2, and 0.02) were spotted onto the medium and grown at 30°C under aerobic conditions, unless otherwise specified. When necessary, the cells were grown under anaerobic conditions using AnaeroPack Anaero sachets (Mitsubishi Gas Chemical Co., Inc.). Appropriate amino acids were removed from SC medium in order to maintain plasmids. SC-NA is SC medium without NA and was prepared as described previously (13) through the dephosphorylation of NMN by alkaline phosphatase; after verification by thin-layer chromatography (TLC) (21), the reaction mixture was filtered through a cellulose acetate membrane (pore size of 0.20 μm; Advantec) and used as NmR. Plasmids and yeast strains used in this study are described in Tables S2 and S3 in the supplemental material. npt1 bna4 and npt1 bna6 cells were obtained on SC medium supplemented with quinolinic acid (QA).

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medium containing 0.1% 5-fluoroorotic acid (FOA) and 3.3 μM NmR by removing YCp::NPT1 from MK2729 (npt1 bna4 YCp::NPT1) and MK2256 (npt1 bna6 YCp::NPT1), respectively. The resulting strains were subsequently maintained in the presence of 3.3 μM NmR.

**Extraction of NAD⁺.** BY4742 wild-type (WT), bna4, bna6, tna1, sum1, and hst1 cells were diluted to 0.7 × 10⁶ cells/ml (A₆₀₀ of 0.1) in SC-NA or SC medium and grown aerobically at 30°C, either for 9 h to reach approximately 0.7 × 10⁷ cells/ml (log phase) or for 24 h to reach approximately 0.7 × 10⁸ cells/ml (stationary phase). Cells were collected by centrifugation (2,000 g for 5 min) at log and stationary phases and frozen at −30°C. The resulting supernatants of the cultures were subjected to liquid chromatography-mass spectrometry (LC-MS) analysis to detect QA as described below. NAD⁺ was extracted from the yeast cells and treated as described previously (22) except that N₂-saturated solution was not used. Two independent cultivations were conducted.

**LC-MS analysis for NAD⁺.** A UPLC/Xevo QTof system (Waters) equipped with an Acquity UPLC BEH amide column (particle size, 1.7 μm; length, 100 mm; internal diameter, 2.1 mm) (Waters) was used. The mobile phase consisted of buffer A (95% acetonitrile [vol/vol], 10 mM ammonium acetate in water, pH 9.0) and buffer B (50% acetonitrile, 10 mM ammonium acetate in water, pH 9.0). Separation was achieved using the following gradient profile: 0 to 8 min, linear gradient from 98:2 buffer A-buffer B (vol/vol) to 15:85 buffer A-buffer B (vol/vol) and 8 to 10 min, isocratic at 2:98 buffer A-buffer B (vol/vol). The column was reequilibrated with 98:2 buffer A-buffer B (vol/vol) for 5 min. The flow rate was 0.5 ml/min, and the injection volume was 3 μl. The total run time was 15 min. Electrospray ionization was performed in positive-ion mode. Intracellular concentration of NAD⁺ was calculated by taking the volume of a haploid BY4742 cell to be 70 μm³, the volume of a kynurenine pathway— or salvage pathway— knockout cell to be 40 μm³ (23), and the A₆₀₀ of 0.1 to correspond to 0.7 × 10⁶ cells/ml.

**LC-MS analysis for QA.** Yeast cells were cultured, and supernatants of the cultures were obtained as described above. Culture supernatant was mixed with an equal volume of 100% methanol and centrifuged at 12,000 g for 10 min. The resulting supernatant was filtered (0.2-μm pore size) and analyzed as follows. A UPLC/Xevo QTof system equipped with an Acquity UPLC BEH C₁₈ column (particle size, 1.7 μm; length, 100 mm; internal diameter, 2.1 mm) (Waters) was used. The mobile phase consisted of buffer A (0.05% formic acid in water) and buffer B (acetonitrile). Separation was achieved using the following gradient profile: 0 to 0.5 min, linear gradient from 99.9:0.1 buffer A-buffer B (vol/vol) to 10:90 buffer A-buffer B (vol/vol) and 0.5 to 4.5 min, isocratic at 10:90 buffer A-buffer B (vol/vol). The column was reequilibrated with 99.9:0.1 buffer A-buffer B (vol/vol) for 4 min. The flow rate was 0.2 ml/min, and the
FIG 2 Released QA aids the growth of \textit{npt1 bna4} cells. Diluted cell suspensions (4.0 μl; \textit{A}_{\text{400}} of 2, 0.2, and 0.02) were spotted onto the medium and grown at 30°C. (A) \textit{npt1 bna4} cells were unexpectedly viable. MK2729 (\textit{npt1 bna4 YCp::NPT1}), MK2025 (\textit{npt1 YCp::NPT1}) (\textit{NAD}⁺ prototroph), and MK2256 (\textit{npt1 bna6 YCp::NPT1}) cells were spotted on SC medium with FOA (+ FOA) or without FOA (- FOA; SC-Ura-Trp). (B) \textit{npt1 bna4} and \textit{npt1 bna6} cells were inviable in the absence of NmR. MK2729 (\textit{npt1 bna4 YCp::NPT1}) and MK2256 (\textit{npt1 bna6 YCp::NPT1}) cells were spotted on FOA medium with 3.3 μM NmR (+ NmR) or without NmR (- NmR). (C) Among 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HA), and QA, only QA aided the growth of \textit{npt1 bna4} cells. \textit{npt1 bna4} cells were spotted on SC-NA medium containing no \textit{NAD}⁺ precursor (none) or the indicated compounds at 3.3 μM.

Injection volume was 3 μl. The total run time was 8.5 min. Electrospray ionization was performed in negative-ion mode.

\textbf{LC-MS analysis for NA.} An LCMS-2010EV system equipped with a Unison UK-Amino (100 by 2.0 mm) column (Imtakt) was used. The mobile phase consisted of buffer A (acetonitrile) and buffer B (0.1% formic acid in water). Separation was achieved using the following gradient profile: 0 to 8 min, linear gradient from 50:50 buffer A-buffer B (vol/vol) to 20:80 buffer A-buffer B (vol/vol), 8 to 10 min, isocratic at 10:90 buffer A-buffer B (vol/vol), and 10 to 18 min, isocratic at 50:50 buffer A-buffer B (vol/vol). The flow rate was 0.2 ml/min, and the injection volume was 1 μl. The total run time was 18 min. Electrospray ionization was performed in positive-ion mode. The lower detection limit of NA was 0.012 μM.

\textit{Quantitative PCR (qPCR).} Yeast cells were grown as for extraction of \textit{NAD}⁺ but immediately frozen in liquid nitrogen after collection of cells. RNA was extracted from the cells using hot phenol (24), treated with DNase I, and purified on an RNeasy column (Qiagen). cDNA was prepared from 0.3 μg of RNA using a ReverTra Ace qPCR RT kit (Toyobo) and analyzed using a LineGene (BioFlux) using the SYBR green Real-time PCR Master Mix (Toyobo) under the following conditions: 1 cycle at 95°C for 1 min and 40 cycles at 95°C for 15 s, 56°C for 15 s, and 72°C for 30 s. The following oligonucleotides were used: ACT1-F, ATGGATTCTGAG GTTGCCT; ACT1-R, CTTCTGCTTCTGGTGTA; BNA4 601_620F, CGGCTACTGAGGAATCTCA; BNA4 827_848R, ACAGCATGCTCCA AATCAT; BNA6 376_395F, ATATCACGTTGGCCTGTC; BNA6 597_616R, CCACCTGATCTTCACAGCA; TNA1 924_943F, CCAAGT GTGGCAAGAGCTA; TNA1 1152_1171R, GACAGAGCAGGAGTG AAAT; NPT1_314_333F, CAGAAGAATTCGAGGGCCCA G; and NPT1_471_492R, GCAGCTCTTCCTCAGGTT.

\textbf{RESULTS}

\textbf{Unexpected viability of \textit{npt1 bna4} cells.} \textit{NAD}⁺ is synthesized via the kynurenine (\textit{de novo}) or salvage pathway (Fig. 1). \textit{S. cerevisiae} cells lacking both \textit{NPT1} and \textit{BNA6} (\textit{npt1 bna6} cells) or \textit{NPT1} and \textit{BNA4} (\textit{npt1 bna4} cells) are inviable on standard medium (YPD or SC medium) containing no NmR because they lack the ability to synthesize \textit{NAD}⁺ although exogenous NmR supports the growth of \textit{npt1 bna6} cells and probably \textit{npt1 bna4} cells (17, 18). Unexpectedly, we observed that \textit{npt1 bna4} cells were viable in a special case, as follows.

We deleted BNA4 and BNA6 from \textit{npt1} cells carrying YCp::NPT1, resulting in MK2729 (\textit{npt1 bna4 YCp::NPT1}) and MK2256 (\textit{npt1 bna6 YCp::NPT1}) cells, respectively. These cells were spotted adjacent to \textit{NAD}⁺ prototrophic MK2205 (\textit{npt1 YCp::NPT1}) cells on SC medium containing FOA (FOA medium) and were expected to be inviable. Unexpectedly, MK2729 (\textit{npt1 bna4 YCp::NPT1}) cells survived on FOA medium (Fig. 2A), which was not in agreement with the previous result that \textit{npt1 bna4} cells are inviable (18). In contrast, MK2256 (\textit{npt1 bna6 YCp::NPT1}) cells were inviable as reported previously (Fig. 2A) (18). Interestingly, when MK2729 (\textit{npt1 bna4 YCp::NPT1}) cells alone were spread on FOA medium, no growth was observed (data not shown). Furthermore, when \textit{NAD}⁺ prototrophic MK2205 (\textit{npt1 YCp::NPT1}) cells were not spotted in adjacent positions, both double mutants were inviable on FOA medium although they were viable on FOA medium containing 3.3 μM NmR (Fig. 2B). The inviability of \textit{npt1 bna4} cells agreed with a previous result (18). Isogenic WT (BY4742) cells also supported the growth of \textit{npt1 bna4} cells but not of \textit{npt1 bna6} cells (data not shown).

The unexpected viability of \textit{npt1 bna4} cells that were adjacent to \textit{NAD}⁺ prototrophic cells reminded us of a recent report demonstrating that \textit{NAD}⁺ prototrophic \textit{S. cerevisiae} cells release NmR, which supports the growth of \textit{npt1 bna6} cells (17). Thus, we speculated that \textit{NAD}⁺ prototrophic cells released some compound (or compounds) that aided the growth of \textit{npt1 bna4} cells, but not that of \textit{npt1 bna6} cells. Because the growth of \textit{npt1 bna6} cells was not supported (Fig. 2A), we considered it unlikely that NmR itself was released. \textit{npt1 bna4} cells on FOA medium were confirmed to require Ura and were maintained in the presence of 3.3 μM NmR.

\textit{QA is a novel \textit{NAD}⁺ precursor.} In the kynurenine pathway, three compounds (3-hydroxykynurenine, 3-hydroxyanthranilic acid, and QA) are intermediates that require Bna4, but not Bna6, for their biosynthesis (Fig. 1B). We sought to determine whether any of all of these compounds are utilized as \textit{NAD}⁺ precursors by \textit{npt1 bna4} cells. As shown in Fig. 2C, only QA aided the growth of \textit{npt1 bna4} cells, suggesting that \textit{NAD}⁺ prototrophic yeast cells could release at least QA, which could support the growth of \textit{npt1 bna6} cells but not that of \textit{npt1 bna4} cells. Secretion of QA into the medium by \textit{S. cerevisiae} WT cells was confirmed by LC-MS (Table 1) although secretion

\begin{table}[h]
\centering
\caption{QA secretion into the medium by \textit{S. cerevisiae} WT cells\textsuperscript{a}
\begin{tabular}{lll}
\hline
Cell type & Initial NA & QA concn (μM)\textsuperscript{b} \\
\hline
Log phase & 0 & 1.6 ± 0.6 \\
& 3.3 & 1.3 ± 0.8 \\
& 33 & 1.3 ± 0.6 \\
Stationary phase & 0 & 3.8 ± 1.2 \\
& 3.3 & 3.6 ± 1.5 \\
& 33 & 3.7 ± 1.0 \\
\hline
\end{tabular}
\textsuperscript{a} Cells were cultivated for 9 h (log phase) or for 24 h (stationary phase), and the concentration of QA in the supernatant of the culture was determined by LC-MS analysis as described in Materials and Methods. QA was not detected in SC-NA medium itself. The mean values ± standard deviations are shown (n = 6).
\textsuperscript{b} Initial concentrations of NA in the medium.
\end{table}

\textbf{TABLE 1}\n
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Intracellular concentrations of NAD\(^+\) were lower in stationary-phase than in log-phase cells (Fig. 4). It is noteworthy that supplementation of NA or QA enhanced intracellular NAD\(^+\) concentrations of WT, bna4, bna6, sum1, and hst1 cells, especially at log phase, but not those of tna1 cells, confirming the role of QA as an NAD\(^+\) precursor and supporting the role of Tna1 as transporter of QA. These measurements also confirmed that intracellular NAD\(^+\) is exhausted during stationary phase.

**DISCUSSION**

This is the first report to demonstrate that *S. cerevisiae* secretes QA into the medium and utilizes extracellular QA that enters the cell, probably via Tna1, as a novel NAD\(^+\) precursor. The physiological relevance of utilization of extracellular QA is evidenced by the observation that extracellular QA enhances the intracellular NAD\(^+\) concentration (Fig. 4). *S. cerevisiae* cells may secrete QA into the medium for storage and reutilization in order to maintain a suitable intracellular NAD\(^+\) concentration although a possibility that QA is excreted as a potentially toxic molecule remains.

In addition, our data would extend previous findings (6, 26). Llorente and Dujon observed that prototrophic diploid cells released some compounds that aided the growth of *bna1* cells, but not of *bna1* tna1 cells, in SC-NA medium; they concluded that NA was released from the prototrophic cells (6). However, because no NA could be detected by LC-MS in the medium (data not shown) and because uptake of low concentrations of QA would be blocked in *tna1* cells (Fig. 3B), we consider it more likely that the prototrophic diploid cells released QA, which aids the growth of *bna1* cells but not *bna1* tna1 cells (Fig. 1B).

Transmission levels of TNA1 and BNA4 are slightly induced in log-phase WT cells grown in SC-NA medium (26), which we also observed in this study (Fig. 4). In contrast, transcription levels of TNA1, BNA4, and BNA6 were suppressed and not induced in WT cells at stationary phase when cells were grown in SC-NA medium, despite their low intracellular NAD\(^+\) concentrations; these genes were highly induced in *bna4* and *bna6* cells, moderately induced in *sum1* and *hst1* cells, and slightly induced in *tna1* cells (Fig. 4). Based on these observations, we speculate that in stationary-phase cells, a high intracellular concentration of NAD\(^+\) is not required; in these cells, the kynurenine pathway in conjunction with secreted QA provides sufficient intracellular NAD\(^+\). Transmission levels of TNA1, BNA4, and BNA6 were suppressed by an unknown mechanism that probably involves Hst1/Sum1 in stationary-phase cells in the presence of a functional kynurenine pathway. However, in the absence of a functional kynurenine pathway, cells respond strongly to situations that cause lower intracellular NAD\(^+\) concentrations, as shown in Fig. 4.

**Intracellular NAD\(^+\) concentrations and transcription of pathway-related genes.** Intracellular concentrations of NAD\(^+\) in log- or stationary-phase WT, *bna4*, *bna6*, *tna1*, *sum1*, and *hst1* cells grown in SC-NA medium supplemented with NA or QA were measured (Fig. 4). Hst1 is an NAD\(^+\)-dependent deacetylase that probably participates in regulation of the kynurenine pathway as a sensor of intracellular NAD\(^+\) concentration, and Sum1 is a transcription factor that binds Hst1 (25, 26).
NmR was identified as a novel NAD⁺ precursor in S. cerevisiae and probably also mammals (12, 13). In this study, QA was also demonstrated to act as a NAD⁺ precursor in S. cerevisiae. TNA1 orthologs are common among the genomes of various fungi. Thus, QA may function as a universal NAD⁺ precursor in fungi except for in a few that lack a BNA6 ortholog, such as C. glabrata, Schizosaccharomyces pombe, and Kluyveromyces lactis (27). Collectively, our data thus shed new light on the significance of QA, which had previously been recognized only as an intermediate in the kynurenine pathway.

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