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Adenine Deaminase and Adenine Utilization in *Saccharomyces cerevisiae*

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Compared with other purine salvage and nitrogen catabolism enzymatic activities, adenine deaminase (adenine aminohydrolase [AAH]; EC 3.5.4.2) activity in *Saccharomyces cerevisiae* is uniquely regulated. AAH specific activity is not induced by adenine and is reduced sevenfold when cells are cultivated in medium containing proline in place of ammonium as the sole nitrogen source. Exogenous adenine enters metabolic pathways primarily via the function of either AAH or adenine phosphoribosyltransferase (APRT; EC 2.4.2.7). Exogenous adenosine cannot normally be utilized as a purine source. Strains efficiently utilized adenosine or inosine when grown in pH 4.5 medium containing Triton X-100. A recessive mutation permitting utilization of adenosine or inosine in standard media was isolated. In both situations, growth of purine auxotrophs required either AAH or APRT activity. With medium containing either ammonium or proline as a nitrogen source, minimum doubling times of purine auxotrophs deficient in either APRT or AAH were measured. In proline-based medium, AAH and APRT permitted equal utilization of exogenous adenine. In ammonium-based medium, the absence of APRT increased the minimum doubling time by 50%. Similar experiments using sufficient exogenous histidine to feedback inhibit histidine biosynthesis failed to affect the growth rates of adenine auxotrophs blocked in AAH or APRT, indicating that the histidine-biosynthetic pathway does not play a significant role in adenine utilization. The gene that encodes AAH in *S. cerevisiae* was isolated by complementation using yeast strain XD1-1, which is deficient in AAH, APRT, and purine synthesis. A 1.36-kb *Eco*RI-*Sph*I fragment was demonstrated to contain the structural gene for AAH by expressing this DNA in *Escherichia coli* under control of the *trp* promoter-operator. Northern (RNA) studies using the AAH-, APRT-, and CDC4-coding regions indicated that AAH regulation was not mediated at the level of transcription or mRNA degradation.

Purine salvage and interconversion pathways are ubiquitous in cellular metabolism (22, 23, 39). Genetic deficiency in these pathways results in human diseases, most notably, severe combined immune deficiency, which is the consequence of adenosine deaminase (ADA; EC 3.5.4.4) or purine nucleoside phosphorylase (EC 2.4.2.1) deficiency. These enzymes constitute one of two pathways in humans that mediate the balance of dGTP and dATP in circulating T cells (25).

Research aimed at treating these disorders has focused on microorganisms as model systems for understanding the functional importance and regulation of the biochemical pathways. Purine salvage, interconversion, and degradation pathways in *Saccharomyces cerevisiae* (Fig. 1) have been studied at both the genetic (3, 8, 28, 34–36) and physiological (3, 5, 9, 21, 31, 36, 37) levels. Adenine or hypoxanthine (but not adenosine, inosine, or guanine) (27) can serve as an exogenous purine source and is readily transported into cells by the product of the *APP1* gene (26). Adenine enters yeast metabolism (5, 36) via two pathways: deamination by adenine aminohydrolase (AAH) into hypoxanthine, followed by conversion into IMP, and conversion by adenine phosphoribosyltransferase (APRT) into AMP, followed by deamination into IMP. Laten et al. (13) reported that APRT- and AAH-deficient yeast cells converted [^{14}C]adenine into [^{14}C]adenosine and [^{14}C]inosine, presumably through the action of nucleoside hydrolase (NH; EC 3.2.2.2) (9) and ADA. The amount of adenine incorporated via this pathway is extremely low (less than 1% of that of either AAH or APRT), consistent with the reported irreversibility of NH and the poor reactivity of adenosine with the reversible activity of purine nucleoside phosphorylase (9). The effectiveness of this pathway for adenine utilization is very poor, as evidenced by the inability of purine auxotrophs deficient in AAH and APRT to grow in media containing adenine as the sole purine source (30).

Histidine biosynthesis yields 5-aminoimidazole-4-carboxylic acid ribonucleotide (AICAR) as a by-product. AICAR is also an intermediate in the purine-biosynthetic pathway. The role of histidine-derived AICAR in the utilization of exogenous adenine is not well understood. This relationship between biosynthetic pathways may aid the balancing of AMP and GMP in vivo.

In this study, seven enzymatic activities modulating the levels of purine derivatives were measured. Experiments evaluating the ADA-dependent pathway by in vitro assay or by in vivo experimentation using adenosine utilization (*aut*) mutants or media permitting uptake of exogenous adenosine are reported. By using strain XD1-1, which requires hypoxanthine for growth, the gene that confers AAH activity was isolated by selection for growth on medium containing adenine as the sole purine source. This gene (*AAH*) was subcloned and demonstrated to encode AAH by expressing the gene in *Escherichia coli*, an organism devoid of this activity (39). Yeast RNA was probed with a portion of *AAH* to determine whether *AAH* mRNA levels change in concert with the observed regulation of AAH.

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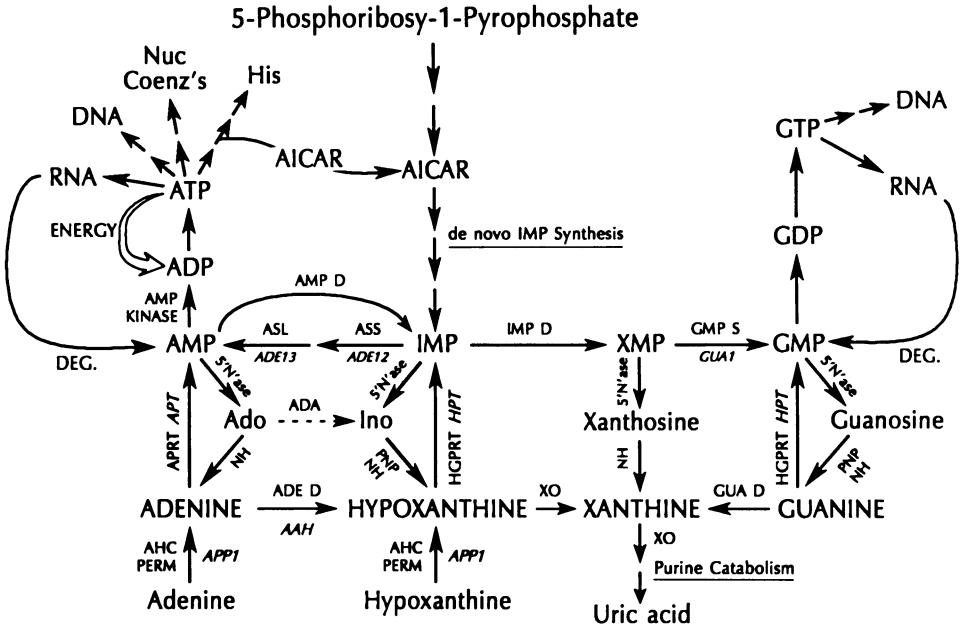

FIG. 1. Schematic representation of purine salvage, interconversion, and degradation pathways in *S. cerevisiae*. See the text for a general discussion. Mutations which block individual reactions are in italics. Abbreviations: ADE D, adenine deaminase (adenine aminohydrolase); AHC PERM, adenine-hypoxanthine-cytosine permease; AMP D, AMP deaminase; ASL, adenylosuccinate lyase; ASS, adenylosuccinate synthetase; DEG., degradation; GUA D, guanine deaminase; GMP S, GMP synthetase; His, histidine; IMP D, IMP dehydrogenase; 5'N'ase, 5' nucleotidase; Nuc Coenz's, nucleotide coenzymes; PNP, purine nucleoside phosphorylase; XO, xanthine oxidase; Ino, inosine; Ado, adenosine.

MATERIALS AND METHODS

Strains and plasmid manipulation. The strains of *S. cerevisiae* and *E. coli* used in this study are listed in Table 1. All genetic manipulations were carried out by using standard techniques (33). Yeast transformations were performed by using the protoplast-producing method of Beggs (4). Gene disruption was performed by the modified transformation procedure described by Rothstein (30).

Ethyl methanesulfonate-mutagenized (15) XV610-8C cells were plated and selected for growth on adenine omission medium (33) supplemented with 100 μg of adenosine per ml in a manner similar to that reported previously (1). Six isolates from separate mutagenesis reactions were characterized. One isolate XV610-8C-V3 was selected for further study.

Restriction enzyme, T4 ligase, and other enzyme reaction conditions were those of the manufacturers. Plasmid preparation, miniscreen analysis, Southern analysis, and Northern (RNA) analysis methods were those of Maniatis et al. (17). Plasmid DNA was isolated from yeast as described by Nasmyth et al. (24) as modified by Sherman et al. (33) and used to transform *E. coli* HB101 or RR1 to drug resistance.

All yeast media were prepared as described by Sherman et al. (33). Nitrogen sources were present at 0.2% (wt/vol). Purine auxotrophs deficient in adenine utilization were routinely supplemented with 10 μg of hypoxanthine per ml, except as noted otherwise. NaOH was used as needed to adjust all media to pH 5.5, except as noted otherwise. *E. coli* was routinely grown in L broth plus the appropriate antibiotic (17). *E. coli* prepared for enzyme assay testing was cultured in M9 minimal-salts medium plus 0.5% acid-hydrolyzed casein, 10 μg of L-tryptophan per ml, and 1 μg of thiamine per ml as previously described (17). All chemicals used were of reagent grade.

A medium allowing purine auxotrophs to utilize adenosine or inosine was devised on the basis of the observations of McCusker et al. (19) and Murakami et al. (21). The medium consists of adenine omission medium (33) adjusted to pH 4.5 with HCl and supplemented with 100 μg of adenosine or inosine per ml, 0.075% (vol/vol) Triton X-100, 2% dextrose, and 2% agar (Difco). Growth rates of wild-type strains or purine auxotrophs on this medium were comparable to those on synthetic complete medium.

Doubling-time measurements. Minimum-doubling-time determinations for strains grown in various media were made by vigorously shaking 20-ml cultures in 125-ml flasks at 30°C. Aliquots taken at timed intervals were diluted, and cell densities were determined in triplicate by using a hemacytometer. A 1-s pulse of sonication was used, when necessary, to eliminate clumps of cells. Minimum doubling times were determined from the slope of standard semilogarithmic plots of cell density versus time. Simple growth curves were observed, with each culture exhibiting exponential growth for 4 to 6 h.

Cell extracts. Fifty-milliliter cultures, grown to the mid-exponential phase in 250-ml flasks and shaken at 30°C, were centrifuged and washed once in 10 ml of 4°C glass-distilled H_2O and once in 5 ml of buffer A (100 mM potassium phosphate [pH 7.0], 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5% [vol/vol] ethanol). Maximal amounts of enzymatic activity were obtained when cells were broken in 2.5 ml of buffer A plus an equal volume of clean, sterilized glass beads (400-μm diameter). Cells were disrupted in 15-ml Corex tubes by vortexing at top speed on a tabletop mixer for 20 periods of 30 s separated by 30-s periods of cooling on ice. The tubes were centrifuged at 10,000 × *g* for 10 min. A 1-ml volume of each supernatant was transferred to a prechilled Eppendorf tube and centri-

TABLE 1. Organisms employed in this study

Strain	Source (reference)	Genotype
S. cerevisiae		
79	L. Hartwell, YGSC[a]	α *leu2-1 trp1-1*
XV610-8C	V. McKay, YGSC	a *ade2 ade6 leu2-1 lys1 trp1-1 can1*
382-31	L. Hartwell, YGSC	α *his4A ura1 met2*
DS10A	R. A. Woods (29)	a *ade2 apt-1 aah-1*
MD105	This study; isolate from cross 79 × DS10A	α *ade2 apt-1 aah-1 leu2-1 trp1-1*
XD1-1	This study; isolate from cross MD105 × XV610-8C	a *ade2 ade6 trp1-1 leu2-1 apt-1 aah-1*
XD3-8	This study; isolate from cross XD1-1 × 382-31	α *his4A ade6 ura1 met2 leu2-1 trp1-1 apt-1 aah-1*
XV610-8C-V3	This study; ethyl methanesulfonate mutagenesis of XV610-8C	a *ade2 ade6 leu2-1 lys1 trp1-1 can1 aut-1*
XD7-2B	This study; isolate from cross XD3-8 × XV610-8C-V3	a *ade6 apt-1 his4A leu2-1 trp1-1 can1 aut-1*
XD7-5B	This study; isolate from cross XD3-8 × XV610-8C-V3	a *ade6 his4A leu2-1 trp1-1 can1 aut-1 ura1*
XD7-6B	This study; isolate from cross XD3-8 × XV610-8C-V3	a *ade6 aah-1 his4A trp1-1 can1 aut-1 ura1*
E. coli		
HB101	ATCC[b] 33694	F^- *hsdS20 recA13 leuB6 thi-1 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-
RR1	ATCC 31343	F^-; same as HB101 except *recA*$^+$

[a] YGSC, Yeast Genetic Stock Center.
[b] ATCC, American Type Culture Collection.

fuged for 10 min at 4°C in a microcentrifuge. Enzymatic activities from the supernatant were found to be stable for at least 10 h. Protein content was determined by the method of Lowry et al. (16) with bovine serum albumin (BSA; fraction V; Sigma Chemical Co.) as the standard. Extracts usually contained 3 to 8 mg of total protein per ml. Samples were diluted for enzyme assays in buffer A plus 5 mg of BSA per ml.

Assays. For each of the assays described, 1 U is defined as 1 nmol of substrate consumed per min. Quantification was made by using radiolabeled substrates and thin-layer chromatography (TLC) separation of products based on the method of Leung and Schramm (14). All exhibited linear enzyme kinetics up to a minimum of 5% substrate conversion (data not shown). The following radioisotopes were purchased from ICN Pharmaceuticals and used as R_f standards and as tracers in reaction mixtures: [8-^{14}C]adenine (56 mCi/mmol), [8-^{14}C]hypoxanthine (54 mCi/mmol), [8-^{14}C]ATP (51 mCi/mmol), [8-^{14}C]AMP (58 mCi/mmol), and [8-^{14}C]adenosine (50 mCi/mmol).

AAH activity (20) was measured in 100-μl (final volume) reactions containing 100 mM potassium phosphate (pH 7.0), 1 mM EDTA, 1.5 mM adenine sulfate, 0.05% BSA, 10 mM 2-mercaptoethanol, and 1 μCi of [8-^{14}C]adenine. Reactions were started by adding 1 to 3 μl of extract or dilution. The reaction tubes were then incubated at 30°C for 30 to 60 min. Reactions were terminated by chilling on ice and rapidly spotting and drying 5 μl of reaction mixture in duplicate onto Polygram cellulose PEI300 sheets (Kodak). Chromatograms were developed in a solvent consisting of 3 parts of *tert*-amyl alcohol, 2 parts of 98% formic acid, and 1 part of H_2O for ~3 h. The sheets were dried and autoradiographed overnight by using Kodak XAR film. Chromatograms and autoradiograms were then aligned, and the portion of the TLC containing hypoxanthine and adenine (R_fs, ~0.5 and ~0.6, respectively) were cut out and placed in 5 ml of scintillation fluid. The quantities of [^{14}C]hypoxanthine and [^{14}C]adenine were determined by using a Beckman LS7500 liquid scintillation counter. The rate of hypoxanthine formation was calculated from the percentage of [^{14}C]purine converted during the reaction period multiplied by the total amount of adenine present in the reaction tube. No hypoxanthine was generated following incubation of the reaction mixture in the absence of cell extracts or reactions containing extracts from cells carrying *aah-1*.

APRT activity was determined in an analogous fashion. The 100-μl reaction mixture (36) contained 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM adenine sulfate, 1 mg of 5′-phosphoribosyl-1-PP_i per ml, 1 mM dithiothreitol, 0.5 mg of BSA per ml, and 1 μCi of [8-^{14}C]adenine. The spots corresponding to AMP (R_f = ~0.25) and adenine were excised and counted.

AMP deaminase (EC 3.5.4.6) and AMP kinase (EC 2.7.4.3) activities were estimated by using the reaction conditions described by Yoshino et al. (38) for obtaining maximal AMP deaminase activity: 10 mM cacodylate buffer (pH 7.1), 50 mM KCl, 20 mM NaCl, 2 mM ATP, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mg of BSA per ml, 10 mM unlabeled AMP, and 0.25 μCi of [8-^{14}C]AMP. Reactions (100 μl) were incubated for 30 min at 30°C. The amount of AMP deaminase activity was determined from the amount of [8-^{14}C]IMP formed. The amount of AMP kinase activity was estimated by determining the combined percentage of ADP and ATP formed during the reaction period. The calculated amount of AMP kinase activity is an underestimate of the actual specific activity. AMP kinase has been previously purified to homogeneity and characterized by Ito et al. (10). The reaction condition employed here is theoretically capable of estimating 70 to 80% (10) of the actual amount of AMP kinase enzyme present.

AMP 5′-nucleotidase (EC 3.1.3.5) activity was estimated from the percentage of [^{14}C]AMP converted to [^{14}C]adenosine (R_f, ~0.55) during 30 min of incubation at 30°C using the following 100-μl reaction mixture: 100 mM Tris-HCl (pH 8.0), 5 mM unlabeled AMP, 0.25 μCi of [8-^{14}C]AMP, 0.5 mg of BSA per ml, and 1 mM dithiothreitol.

NH (9) was measured in 100-μl reactions containing 100 mM potassium phosphate (pH 7.0), 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mg of BSA per ml, 10 mM unlabeled adenosine, and 0.25 μCi of [8-^{14}C]adenosine. The presence of phosphate in the reaction did not affect the activity of NH from crude extracts (9; unpublished data).

Hypoxanthine-guanine phosphoribosyltransferase [H(G)PRT] (EC 2.4.2.8) activity (32) was measured by using the

same reaction conditions (35) as for the APRT assay with the substitution of unlabeled hypoxanthine and [8-^{14}C]hypoxanthine for adenine. Chromatography of hypoxanthine and IMP on TLC sheets resolved the compounds to R_f values of ~0.5 and ~0.2, respectively.

Isolation and analysis of mRNA. Cell culture volumes of 100 to 300 ml were grown to the mid-exponential phase with rapid shaking at 30°C, centrifuged for 5 min at 5,000 × *g*, washed with 0.5 volume of ice-cold glass-distilled H_2O (all aqueous solution was treated with diethylpyrocarbonate to inactivate RNase activity), centrifuged, and suspended in 5 ml of RNA extraction buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 5 mM EDTA, 1% sodium dodecyl sulfate). Total nucleic acid was extracted by adding 5 ml of phenol-chloroform-isoamyl alcohol (24:24:1) plus 10 g of baked glass beads (400-mm diameter) and then vortexed at top speed (15 periods of 30 s each separated by 30-s periods of cooling on ice). Following centrifugation, the aqueous phase was re-extracted twice with 5 ml of phenol-chloroform-isoamyl alcohol and once with an equal volume of chloroform. The nucleic acid in the aqueous phase was then precipitated with 0.1 volume of 3 M sodium acetate (pH 5.6) and 2.5 volume of ethanol. The nucleic acid was pelleted by centrifugation for 30 min at 10,000 × *g*, washed once in 5 ml of 80% ethanol, and dried in vacuo. Chromosomal DNA was removed, and poly(A)$^+$ RNA was enriched by chromotography on oligo(dT) cellulose (P.L. Biochemicals) as previously described (17).

RNA samples were treated with glyoxal, electrophoresed, transferred to nitrocellulose, and probed as described by Maniatis et al. (17). Restriction fragments were prepared as probes by nick translation by using α-[^{32}P]ATP (~2,000 Ci/mmol) to a radiospecific activity of 1 × 10^8 to 10 × 10^8 cpm/μg. Each probe was used at 5 × 10^5 cpm/ml of hybridization solution.

RESULTS

Utilization of adenine and adenosine. Adenine was readily incorporated into yeast cellular metabolism via AAH or APRT. These pathways were demonstrated by using TLC and autoradiography of products from reactions containing [^{14}C]adenine and extracts of mutant yeast strains alternatively deficient in either or both of these pathways (Fig. 2). Lane 1 demonstrates absence of adenine conversion in the absence of active AAH or APRT. The presence of AMP in lanes 2 and 4 revealed APRT activity. The presence of hypoxanthine in lanes 3 and 4 demonstrated the action of AAH. Under these reaction conditions, some hypoxanthine was converted into IMP, a reaction product of H(G)PRT.

A third potential pathway, involving direct conversion of adenine into adenosine, could not be demonstrated in vitro when radiolabeled adenine was incubated in extracts containing either 1 mM ribose or ribose-1-PO_4. The lack of detectable radiolabeled adenosine as a reaction product under a variety of assay conditions, including conditions which detected the reversible purine nucleoside phosphorylase activity found in *E. coli* (12), indicated that *S. cerevisiae* contains minimal activity of this type (<1 nmol of adenine converted per min per mg of protein). Direct conversion of radiolabeled adenosine into inosine or AMP could not be revealed by using a variety of reaction conditions, including those that did detect ADA activity of *E. coli* (29) and *Aspergillus oryzae* (unpublished data) and conditions that Marmocchi et al. (18) and Yoshino et al. (38) used to detect ADA from commercial baker's yeast (*S. cerevisiae*). The data indicated that these *S. cerevisiae* cell extracts contained minimal or no ADA and adenosine kinase activities.

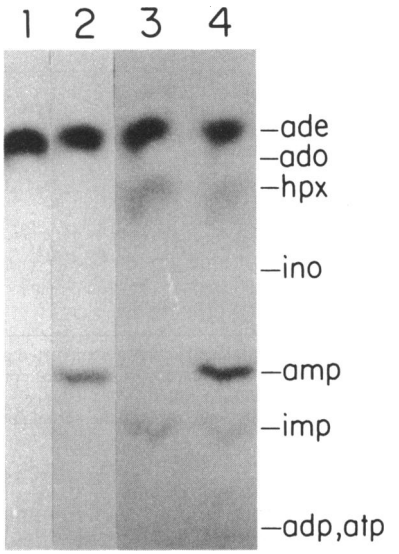

FIG. 2. TLC of [8-^{14}C]adenine and products of APRT and AAH in vitro. Cell extracts were prepared from XD3-8 (*aah1-1 apt1-1*; lane 1), XD7-6B (*aah1-1*; lane 2), XD7-2B (*apt1-1*; lane 3), and XD7-5B (lane 4) and incubated in APRT reactions. AAH was submaximally active under these conditions. Abbreviations: ade, adenine; ado, adenosine; adp, ADP; amp, AMP; atp, ATP; hpx, hypoxanthine; imp, IMP; ino, inosine.

Because in vitro measurements are, by their nature, indirect measurements of in vivo processes, a means was sought to test for biologically significant ADA. Although wild-type *S. cerevisiae* cannot normally utilize adenosine as a purine source (1, 27), a low-pH medium containing 0.075% Triton X-100 which permitted utilization of adenosine and inosine was devised. The hypothesis that *S. cerevisiae* lacks a functional pathway for conversion of adenine into inosine via adenosine was corroborated by observing the inability of purine auxotrophs to grow in this medium containing adenosine as the sole purine source when both APRT and AAH were genetically eliminated (XD1-1). Normal growth was observed when inosine was added to this medium or the strain carried *AAH* (XD7-2B) or *APT* (XD7-6B).

Anderson and Roth (1) described recessive mutations which permitted *S. cerevisiae* to utilize exogenous adenosine as the sole purine source. These mutations increased cell permeability to adenosine approximately 10-fold. A mutation of this type was sought for this study. XV610-8C-V3 was isolated following mutagenesis by ethyl methanesulfonate and selection on adenine omission medium containing 100 μg of adenosine per ml. XV610-8C-V3, but not XV610-8C, was able to utilize inosine as well. XV610-8C-V3 was mated with XD3-8. The diploid was not able to grow on plates containing adenosine as the sole purine source. Examination of progeny from the sporulated diploid revealed a 2:2 segregation pattern of the adenosine utilization phenotype, indicating the generation of a single recessive mutation hereby assigned the designation *aut-1*. Progeny able to utilize adenine were also able to utilize adenosine. None of the 12 *aah apt* isolates obtained were able to utilize adenosine for growth.

Effect of nitrogen source on the enzymes of the adenine salvage pathway. AAH and APRT specific activities were measured in cell extracts of purine prototrophic strain 79 (α

TABLE 2. Effects of different growth media on purine interconversion enzyme activity[a]

Enzyme	Mean sp act ± SD				
	Proline minimal medium	Glutamate minimal medium	Ammonium minimal medium	Synthetic complete medium	YEPD
AAH	14.3 ± 2.9	107 ± 10.4	108 ± 10.5	142 ± 13.1	136.0 ± 13.0
APRT	39.0 ± 7.8	52 ± 9.3	59 ± 9.6	60 ± 9.6	60.0 ± 9.6
AMP deaminase	42 ± 8.0	67 ± 9.8	70 ± 9.9	66 ± 9.8	67.0 ± 9.8
AMP kinase[b]	346 ± 24.2	109 ± 15.3	140 ± 10.2	40 ± 7.9	44.0 ± 8.1
5′ nucleotidase	14 ± 2.8	9.7 ± 2.2	3.8 ± 0.8	7.5 ± 1.8	3.7 ± 0.8
NH	5.0 ± 1.0	<1	<1	<1	<1
H(G) PRT	15 ± 2.9	18 ± 3.3	11.0 ± 2.4	11.0 ± 2.4	11.0 ± 2.4

[a] All values are given as nanomoles of substrate converted per minute per milligram of total protein and represent three experiments each. Strain 79 was grown to the mid-exponential phase in yeast carbon base (Difco) containing the specified nitrogen source (0.2%, wt/vol), 100 μg of adenine per ml, 20 μg of L-leucine per ml, and 20 μg of L-tryptophan per ml.

[b] The values presented are only 70 to 80% of the actual specific activities present, owing to assay limitations (10); see Materials and Methods.

leu2-1 trp1-1) grown in ammonium-based minimal medium in the presence or absence of 100 μg of adenine per ml. Because AAH expression is reportedly affected by ammonium as a nitrogen source (36), enzyme activities of cells grown in media containing the nonrepressive nitrogen sources proline and glutamate (6) were also measured (Table 2). The experiment demonstrated a sevenfold decrease in AAH specific activity when cells were grown in proline-based medium compared with medium containing glutamate or ammonium. APRT specific activity was relatively unaffected. For each of these media, inclusion of adenine did not affect the level of either enzyme.

To determine the regulatory pattern of the other primary purine salvage enzymes that affect adenine utilization, strain 79 was grown to the mid-exponential phase in media varying in nitrogen sources and complexity (Table 2). Each medium was supplemented with 100 μg of adenine per ml. Cell extracts were assayed for 5′ nucleosidase, AMP deaminase, AMP kinase, H(G)PRT, NH, APRT, and AAH. Of these seven enzymes, only AAH exhibited significantly lower specific activity when the strain was grown in proline-based medium. APRT and H(G)PRT exhibited a less-than-twofold change in activity, suggesting that their levels were constitutively expressed. AMP kinase exhibited a ninefold increase in activity when cells were grown in a poor (proline-based minimal) medium rather than a rich medium (YEPD). 5′ nucleotidase exhibited a similar trend, spanning a fourfold range, as did NH, which was detectable only in the proline-based medium. The level of AMP deaminase enzyme in the extract did not significantly change. However, its actual in vivo enzymatic activity is known to be allosterically regulated severalfold (37, 38).

Growth rate measurements of *apt* and *aah* purine auxotrophs. Wild-type *S. cerevisiae* grew very slowly on media containing adenine as the sole nitrogen source (data not shown). Addition of a second nitrogen source, such as proline or ammonium, dramatically increased the growth rate. To ascertain the relative contributions of the APRT and AAH pathways to utilization of adenine as a purine source in vivo, minimum doubling times of strains were determined in proline- and ammonium-based minimal media containing limiting adenine (5 μg/ml). Three purine synthesis-deficient (*ade6*) strains, XD7-5B (*APT AAH*), XD7-2B (*apt-1 AAH*), and XD7-6B (*APT aah-1*), were studied. These strains exhibited essentially equivalent minimum doubling times with proline as the nitrogen source (230 to 245 ± 12 min). However, when ammonium was supplied as the nitrogen source the *apt-1 AAH* strain (XD7-2B) exhibited a significantly longer doubling time (368 ± 18 min) than the other strains (220 to 232 ± 12 min) and XD7-2B grown in proline medium (230 ± 12 min). Although XD7-2B exhibited a longer minimum doubling time in this medium, the culture achieved the same final cell density as the other cultures.

A similar experiment was performed by using purine auxotrophic strain XV610-8C (*APT AAH*) in the presence of increasing concentrations of exogenous histidine (0 to 1.5 mM L-histidine). The cells were grown in histidine omission medium. A set of parallel cultures additionally supplemented with hypoxanthine (20 μg/ml) were incubated for comparisons of growth rates. No significant differences in growth rate or final cell density were observed. The experiment was repeated by using purine auxotrophic strain XD1-1 (*aah-1 apt-1 leu2-1*), bearing either plasmid pCG2, which confers AAH, or pCG3, which confers APRT (the plasmids are described below), with XD1-1 bearing the empty vector CV13 serving as a control. Leucine was omitted from the above-described media to maintain selection for the plasmids. XD1-1 bearing CV13 failed to grow in the absence of hypoxanthine. The presence of hypoxanthine or histidine did not significantly affect the growth rates of the other strains.

Isolation of the AAH structural gene by functional complementation. Strain XD1-1, a *ade2 ade6 trp1-1 leu2-1 apt-1 aah-1*, was transformed with a library of *S. cerevisiae* genomic DNA prepared by K. Nasmyth. The library was prepared by partial *Sau*3A digestion (24), followed by ligation into the unique *Bam*HI site of *LEU2*-bearing yeast shuttle vector CV13 (33). Approximately 90% of the library contained inserts with an average size of 10 kb (24). Transformants were coselected for leucine prototrophy and the presence of APRT or AAH by plating on synthetic leucine omission medium containing adenine. From the estimated 250,000 Leu^+ transformants, approximately 450 colonies were observed on this medium.

Transformants were tested for simultaneous loss of plasmid-borne *LEU* and APRT or AAH activity following growth in nonselective medium, YEPD. Total yeast DNA was prepared (24) from 30 strains which satisfied this criterion and used to transform *E. coli* HB101 (*recA*). Miniscreen analysis of these *E. coli* strains indicated two classes of plasmid. Two plasmids from each class were selected for further study and designated pCG1 through pCG4.

Transformation of XD1-1 with these four plasmids yielded adenine-utilizing strains with high efficiency. Representative colonies from these transformations were colony purified

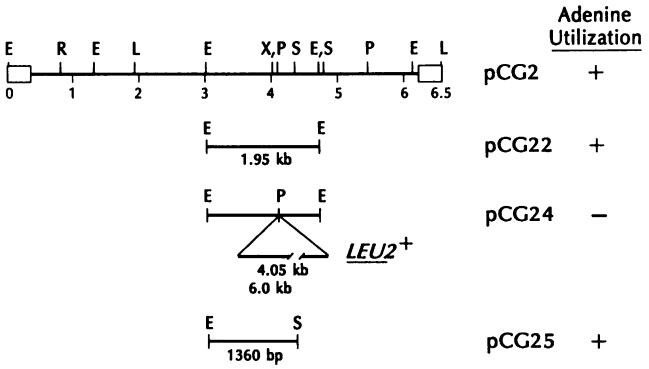

FIG. 3. Localization of *AAH* within the pCG2 restriction map. The restriction map of pCG2 is presented indicating the 6.1-kb genomic fragment inserted at the unique *Bam*HI site of CV13. The open boxes correspond to vector DNA. Restriction fragments from pCG2 were subcloned into appropriately cut YRP7 yeast shuttle vectors bearing *TRP1* (33). Each of the plasmids was tested for conferral of AAH biological activity in *S. cerevisiae* by selecting for growth of transformants on medium containing adenine as the sole purine source. Abbreviations: E, *Eco*RI; L, *Sal*I; P, *Pst*I; X, *Xba*I. pCG24 differs from pCG22 by insertion of a 4.05-kb *Pst*I fragment containing the *LEU2* gene from CV13 at the *Pst*I site within the 1.95-kb *Eco*RI insert.

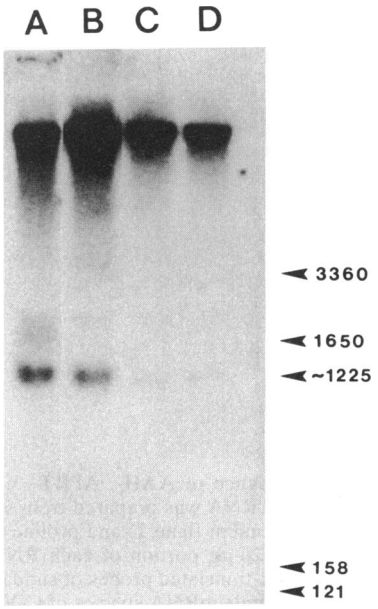

FIG. 4. Northern analysis of AAH-specific mRNA of yeast strain 79 grown in synthetic and rich media. Total nucleic acid was prepared as described in Materials and Methods. The filter was probed with a nick-translated *Eco*RI-*Sph*I (1,360 bp) fragment from pCG25. rRNAs (25S, 18S, 5.8S, and 5S) were used as size markers, and their lengths are presented in nucleotides on the right. Each lane contained 20 μg of nucleic acid from strain 79 grown in proline-based minimal medium (A), ammonium-based minimal medium (B), synthetic complete medium containing ammonium sulfate as the nitrogen source (C), or YEPD (D).

and used to inoculate synthetic complete medium lacking leucine and containing ammonium sulfate as the nitrogen source. Cultures were grown to the mid-exponential phase and assayed for AAH and APRT activities. pCG1 and pCG2 conferred 2- to 3-fold-elevated levels of AAH activity (300 to 350 nmol/min/mg) on XD1-1 over that found in strain 79, while pCG3 and pCG4 conferred 8- to 10-fold-elevated levels of APRT activity (450 to 500 nmol/min/mg).

Conferral of AAH activity was localized to a 1.36-kb *Eco*RI-*Sph*I fragment by subcloning portions of the pCG2 insert (Fig. 3). A 4.05-kb *Pst*I restriction fragment containing the *LEU2* structural gene isolated from CV13 was ligated into the *Pst*I site of the 1.95-kb *Eco*RI insert of pCG22. The resulting plasmid, pCG24, failed to confer the adenine utilization phenotype upon transformation into XD1-1. A gene replacement experiment (30) was performed by using an *Xba*I-*Hin*dIII fragment isolated from pCG24 spanning the inactivated adenine utilization phenotype-conferring region and used to transform haploid strain XD7-6B (*apt-1 AAH*) to leucine prototrophy. Approximately one-half of the tested transformants lost the ability to utilize adenine. Genomic DNAs from several of these isolates were prepared (33), digested with *Eco*RI, and probed by the method of Southern (17) with the 1.36-kb *Eco*RI-*Sph*I fragment from the pCG25 insert. As expected, the wild-type 1.95-kb *Eco*RI fragment had been replaced with the predicted 3.2- and 2.8-kb *Eco*RI fragments from the insert within pCG24 (data not shown).

These experiments indicated that the cloned gene, *AAH*, on pCG22 contained either the AAH structural gene or an obligatory positive regulatory element conferred by the *AAH* locus. To distinguish these possibilities, expression of this gene was sought in *E. coli*, an organism naturally deficient in AAH (39). Extracts of RR1 bearing pCG22 were assayed for AAH activity without success. To ensure expression of the gene in the bacterium, the 1.36-kb *Eco*RI-*Sph*I fragment from pCG25, presumed to contain the AAH-coding region, was ligated adjacent to the indoleacrylic acid-inducible *E. coli trp* promoter-operator of pDR720 (P.L. Biochemicals). Transformants were induced and assayed for AAH activity. A two- to threefold increase (8.0 versus 3.3 nmol/min/mg) was observed when the *Sph*I end was juxtaposed to the *trp* promoter-operator. No induction of AAH activity (0.8 nmol/min/mg) was observed when the opposite orientation was used.

Analysis of AAH mRNA. To determine the size of natural AAH-specific transcripts, strain 79 RNA was examined by Northern analysis (17). Four growth conditions were examined. Total nucleic acid from cells grown in (i) synthetic minimal medium with proline as the sole nitrogen source, (ii) synthetic minimal medium with ammonium as the nitrogen source, (iii) synthetic complete medium (33), or (iv) YEPD were prepared and analyzed. The 1.95-kb *Eco*RI fragment from pCG22 was used as the probe. In each case, a single mRNA transcript, ~1,225 nucleotides long, was observed (Fig. 4). The samples from cells grown in synthetic complete medium and YEPD contained significantly less of the ~1,225-nucleotide species than did the samples from synthetic minimal media. This may result from the greater percentage of rRNA in total cellular RNA that can occur when cells are grown in richer media. A darker band of higher-molecular-weight material, migrating in the position usually associated with contaminating chromosomal DNA, was also observed.

To determine the involvement of steady-state mRNA levels in regulation of expression, poly(A)$^+$ mRNA preparations from two of the four cultures of strain 79 described above were obtained by oligo(dT) chromatography. This procedure enriched the poly(A)$^+$ fraction approximately 10-fold and eliminated contaminating DNA. A sample (20

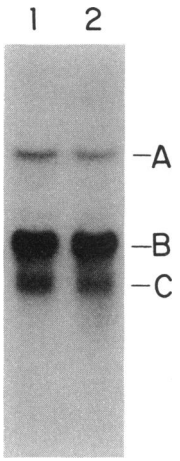

FIG. 5. Relative abundance of AAH-, APRT-, and CDC4-specific transcripts. Poly(A)$^+$ RNA was prepared from strain 79 grown in media containing ammonium (lane 1) and proline (lane 2) as the sole nitrogen sources. A 20-μg portion of each RNA sample was probed by using three nick-translated probes of similar radiospecific activity. A, ~2,600-nucleotide mRNA species of CDC4; B, ~1,225-nucleotide mRNA species of AAH; C, ~825-nucleotide mRNA species of APRT.

μg) of each of the poly(A)$^+$ RNAs was studied by Northern analysis by simultaneously hybridizing the sample with nick-translated probes from three distinct genes: *APT*, *CDC4*, and *AAH*. APRT enzyme levels exhibited a less-than-twofold change in expression under these culture conditions. The APRT-specific probe was a 430-bp *Hin*dIII fragment from pCG3, internal to the coding region (unpublished data). CDC4 is a component of the yeast spindle pole body and reported to be constitutively expressed at 1 to 10 copies per cell (36a). The *CDC4*-specific probe was a 1,950-bp *Bam*HI-*Hin*dIII fragment, internal to the coding region, from pCDC4-71. The AAH-specific probe was the 1,075-bp *Eco*RI-*Pst*I fragment from pCG22, containing a portion of the coding region and an unknown amount of the 3′ noncoding region of AAH. APRT mRNA migrates with an approximate size of ~825 nucleotides (unpublished data). Transcription of *CDC4* results in a ~2,600-nucleotide mRNA. Inspection of the autoradiogram (Fig. 5) indicated that the relative steady-state levels of the three transcripts remain essentially constant, despite the change in the nitrogen source, which effects a sevenfold change in AAH specific activity.

DISCUSSION

Adenine is utilized primarily through the action of AAH and APRT. Commercial baker's yeast exhibits the capacity to convert adenine into adenosine (13) and to deaminate adenosine into inosine (18); however, this pathway was inefficient compared with the AAH and APRT pathways, presumably owing to the poor conversion rate of adenine into adenosine (9, 13). Marmocchi et al. (18) have reported the partial purification of ADA from commercial baker's yeast. The demonstration of ADA in *S. cerevisiae* confirms previous hypotheses of its existence, based upon the kinetics of inosine formation from exogenous adenine in vivo (5, 13) and the report of very low levels of ADA activity in crude cell extracts (38). The involvement of ADA in balancing adenine and guanine derivatives in vivo has been suggested (5); however, direct study of this pathway has been complicated by the inability of wild-type yeast to incorporate exogenous adenosine (1) and the requirement of either APRT or AAH for growth of purine auxotrophs (36).

In this study, conversion of [^{14}C]adenine into [^{14}C]adenosine and [^{14}C]inosine with crude cell extracts was not detected. The discrepancy between this finding and that of Laten et al. (13) could be due to greater sensitivity of the latter's approach. The facts that (i) *apt aah* purine auxotrophs did not utilize adenine as a purine source, (ii) *apt aah* purine auxotrophs did not utilize adenosine as a purine source when grown in medium allowing utilization of adenosine or inosine, and (iii) *aut* strains cannot utilize adenosine unless the strain carries either *APT* or *AAH* support the conclusion that ADA constitutes a minor pathway, not sufficiently efficient to permit growth.

Exogenous histidine at concentrations up to five times (1.5 mM) that required to inhibit (28) 90% of histidine biosynthesis (0.3 mM) had no effect on purine auxotrophs dependent upon either APRT, AAH, or both for growth, compared with cells supplemented with hypoxanthine. This result argues that the histidine biosynthesis byproduct AICAR does not play a major role in mediating conversion of AMP to IMP under these conditions. To simplify analysis of the AAH and APRT pathways for utilization of exogenous adenine, subsequent growth rate experiments employed strains blocked in histidine biosynthesis (*his4A*) prior to AICAR formation.

Minimum-doubling-time measurements indicated that the APRT and AAH pathways were equally capable of incorporating adenine as the sole source of purine when cells were grown in proline-based medium. However, when cells were grown in ammonium-based medium, APRT-deficient cells grew significantly more slowly. One explanation may lie in the relative specific activities of AAH and H(G)PRT when cells were grown with ammonium rather than proline. In proline-based medium, the specific activities of the two enzymes were essentially equal (Table 2). In ammonium-based medium, H(G)PRT specific activity was 10-fold less than that of AAH. This hypothesis is not wholly satisfying in that both proline- and ammonium-based media were capable of supporting equivalent doubling times of *APT* strains and that the specific activity of H(G)PRT was not substantially lower in ammonium-containing medium than in proline-based medium and, therefore, should not be growth rate limiting. One could hypothesize that the accumulating hypoxanthine was excreted, causing a delay in its utilization, a phenomenon of adenine auxotrophs during sporulation (11); however, vegetative purine prototrophs do not normally excrete purines (2). Additional experimentation is needed to ascertain which step limits the growth rate under these conditions before this observation can be explained.

In vitro measurements of the adenine salvage pathway specific activities from cell extracts correlated well with steady-state levels of purine compounds determined in vivo with radiolabeled purine (5). Of the enzymes assayed, only AAH exhibited decreased activity in extracts when cells were cultured in minimal media employing proline as the sole nitrogen source. This decrease in expression, coupled with the lack of inducibility with adenine, indicated that AAH was not subject to nitrogen catabolite repression. Implicitly, then, AAH does not serve primarily as a nitrogen scavenger. The principal role of this enzyme must be to modulate adenine and hypoxanthine levels upon changes in nitrogen availability.

The range of intracellular AAH activity (14 to 140 nmol/min/mg) spans the constitutive level of APRT (40 to 60

nmol/min/mg). Thus, a change in the level of AAH would shift the ratio of exogenous adenine converted into AMP and hypoxanthine. AMP is an important regulatory molecule in *S. cerevisiae* (3). Regulation of AAH may improve the ability of a cell to coordinate nitrogen availability with glycolysis. Logically, a reduction in the availability of nitrogen should be accompanied by a reduction in the flow of carbon through glycolysis (37). AMP and ATP levels may act as signals that mediate the requisite balancing of metabolism by acting through AMP deaminase, an enzyme allosterically inhibited by AMP and stimulated by ATP (38). It has been proposed (37) that increasing the rate of AMP deamination can lead to increases in the rate of glycolysis in *S. cerevisiae*. When cells are grown with a poor (proline) nitrogen source (6), the level of AAH decreases, thereby funnelling exogenous adenine through the APRT-dependent pathway. Increasing the potential rate of AMP formation, by shifting the ratio of AMP to hypoxanthine formed from exogenous adenine, may permit more efficient regulation of cellular energy metabolism.

Consistent with the importance of AMP as a regulatory molecule, it is interesting that the specific activity of AMP kinase increased ninefold (346 versus 42 nmol/min/mg) under these same conditions. Given adequate ATP levels, AMP kinase (10) would convert much of the accumulating AMP into ADP. The resulting effect would be to decrease the degree of inhibition of AMP deaminase and increase the rate of glycolysis. Taken together, the dynamic balance of AMP and ATP is affected by the rates of AMP formation and consumption as exerted by the concentration of these nitrogen source-regulated enzymes and the availability of adenine. The observation that the absolute level of AMP deaminase in extracts was unaffected by the source of nitrogen is consistent with its proposed role as a mediator for this metabolic signal.

Nitrogen deprivation is a strong inducer of sporulation (7). One might hypothesize that the decrease of AAH activity in periods of poor nitrogen availability is important in funnelling adenine into nucleotide formation. This is consistent with gross characterizations of the cell during sporulation. During the first 6 to 7 h, the permeability of the cell to purine influx greatly decreases. This occurs at the same time as the demand for nucleotide precursors steadily increases (7). Jakubowski and Goldman (11) have reported that *S. cerevisiae* excretes large amounts of purine compounds during sporulation in a temporal sequence, beginning with purine bases. Data from both purine auxotrophs and prototrophs support the conclusion that purines are preferentially reutilized from excreted compounds over the internal conversion of either degraded products or de novo IMP. APRT, AAH, and H(G)PRT were shown to be important to sporulation efficiency. Further study of the role of AAH regulation in sporulation may provide insight into the observed regulation of this enzyme by nitrogen availability.

The mechanism that mediates sevenfold regulation of AAH activity remains elusive. Northern experiments failed to detect a comparable change in the steady-state level of AAH-specific mRNA, relative to the constitutively expressed APRT-specific and *CDC4* mRNAs, when cells were grown in media that exert this effect on AAH specific activity. This result rules out the possibility that AAH expression is regulated by a simple transcriptional control mechanism. In light of the fact that AMP deaminase exhibits extensive allosteric regulation (37, 38), the possibility of allosteric regulation of AAH should be thoroughly examined. It is worth noting that in vitro measurements of AAH in yeast extracts readily reflected the nitrogen source-induced regulation of this activity, suggesting that the concentration of AAH enzyme, rather than hypothetical allosteric regulators, primarily affected the level of in vivo activity. Given these findings, one might suspect that translation of AAH-specific mRNA is involved in regulating AAH expression. Further work should focus on determining the mechanism that mediates AAH activity regulation. Use of the cloned AAH gene will facilitate this study.

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