Characterization of Two 5-Aminoimidazole-4-carboxamide Ribonucleotide Transformylase/Inosine Monophosphate Cyclohydrolase Isozymes from Saccharomyces cerevisiae*

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The Saccharomyces cerevisiae ADE16 and ADE17 genes encode 5-aminoimidazole-4-carboxamide ribonucleotide transformylase isozymes that catalyze the penultimate step of the de novo purine biosynthesis pathway. Disruption of these two chromosomal genes results in adenine auxotrophy, whereas expression of either gene alone is sufficient to support growth without adenine. In this work, we show that an ade16 ade17 double disruption also leads to histidine auxotrophy, similar to the adenine/histidine auxotrophy of ade3 mutant yeast strains. We also report the purification and characterization of the ADE16 and ADE17 gene products (Ade16p and Ade17p). Like their counterparts in other organisms, the yeast isozymes are bifunctional, containing both 5-aminoimidazole-4-carboxamide ribonucleotide transformylase and inosine monophosphate cyclohydrolase activities, and exist as homodimers based on cross-linking studies. Both isozymes are localized to the cytosol, as shown by subcellular fractionation experiments and immunofluorescent staining. Epitope-tagged constructs were used to study expression of the two isozymes. The expression of Ade17p is repressed by the addition of adenine to the media, whereas Ade16p expression is not affected by adenine. Ade16p was observed to be more abundant in cells grown on nonfermentable carbon sources than in glucose-grown cells, suggesting a role for this isozyme in respiration or sporulation.

5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR)1 transformylase catalyzes the ninth step of the de novo purine biosynthesis pathway. This reaction involves the formylation of AICAR using 10-formyltetrahydrofolate as the formyl donor (Fig. 1). Saccharomyces cerevisiae possesses two genes, ADE16 and ADE17, that encode AICAR transformylase enzymes that share 85% identity (1). This reaction is the only purine biosynthesis step that is catalyzed by separate isozymes in yeast. Expression of either ADE16 or ADE17 alone is sufficient to support the growth of yeast in adenine-free media, but disruption of both genes results in adenine auxotrophy (1). AICAR transformylase assays of yeast crude extracts demonstrated that the ADE17 gene product is the more active of the two enzymes, but the low level of activity supplied by the ADE16 gene product is sufficient for wild-type growth rates. Given these results, we began to question why yeast possess two AICAR transformylase isozymes and whether these two enzymes have separate metabolic roles in the cell.

Recent research has shown a difference in the regulation of the expression of ADE16 and ADE17 by adenine. Like other purine biosynthesis genes, optimal expression of ADE17 is dependent on the transcription factors Bas1p and Bas2p, and ADE17 expression is repressed by adenine (2). The ADE17 gene is one of the most strongly repressed purine biosynthesis genes, as shown using ADE17-lacZ fusion constructs. Unlike other purine biosynthesis genes, there are no consensus Bas1p and Bas2p binding sequences in the promoter region of the ADE16 gene, and Northern blot analysis has revealed that ADE16 expression is not affected by adenine (2). In this work, His-tagged Ade16p and Ade17p were purified and their activities were characterized, the cellular localization of the two isozymes was determined, and the regulation of Ade16p and Ade17p expression was investigated. The effect of adenine on Ade16p and Ade17p expression was followed at the protein level, using epitope-tagged versions of ADE16 and ADE17 integrated into the yeast genome. The levels of epitope-tagged Ade16p and Ade17p were also monitored in cells grown under various medium conditions in order to elucidate any other differences in the expression of these two isozymes.

EXPERIMENTAL PROCEDURES

Materials—Dimethyl suberimidate, AICAR, and 5-formyltetrahydrofolate were purchased from Sigma. Restriction enzymes and T4 DNA ligase were purchased from Life Technologies, Inc. 5,10-Methenyltetrahydrofolate was prepared from 5-formyl-THF by the method of Rabino nowitz (3). (6R,8S)-10-Formyltetrahydrofolate was prepared from 5,10-methenyltetrahydrofolate according to the procedure of Rowe (4).

Strains and Growth Conditions—The S. cerevisiae strains used in this paper are shown in Table I. Rich media contained 1% yeast extract and 2% Bacto-peptone (DIFCO). Synthetic minimal medium contained 0.7% yeast nitrogen base without amino acids (DIFCO) and was supplemented with the following amino acids as indicated (final concentration): t-serine (375 mg/liter), t-leucine (30 mg/liter), t-histidine (20 mg/liter), t-tryptophan (20 mg/liter), t-methionine (20 mg/liter), uracil (20 mg/liter), and adenine (20 mg/liter). Cells were grown with 2% glucose unless otherwise indicated. Some experiments required the addition of 5-fluoroorotic acid (5-FOA) to agar plates. 5-FOA plates contained 0.7% yeast nitrogen base, 0.1% 5-FOA, 50 mg/liter uracil, 2% glucose, and other amino acids necessary for growth at the concentrations listed above. Yeast transformation was performed using a lithium acetate method (5). Escherichia coli XL1-Blue (Strategene) was used as host for plasmid manipulations. E. coli transformations were performed either by electroporation or by using a high efficiency chemical method
of THF at and concentration of Ade17p was carried out in the same manner as by sonication. After centrifugation at 11,000
grown in a shaking incubator at 37 °C for 3 h. After induction, cells were
thiogalactopyranoside to a final concentration of 1.0 mM. The cells were
extracts (15–30
of 10-formyl-THF and THF at 298 nm, 19.7
for buffer exchange (into 50 mM sodium phosphate, 300 mM NaCl, 1 mM
resulting PCR product and the pET16b vector (Novagen) were cleaved
with NdeI and BamHI. The ADE15 gene was ligated into the vector using T4 DNA ligase to generate the pET16b-ADE16 plasmid for expression of N-terminal histidine-tagged Ade16p. The pET16b-ADE17 construct was prepared in a similar way using oligonucleotide primers, NdeATIC2 and BamATIC2. His-tagged ADE16 and ADE17 were subsequently cloned into the yeast expression vector, pVT-101U (9) to generate the plasmids pVT-HisADE16 and pVT-HisADE17. These constructs were transformed into the ATY3.1 yeast strain to test the ability of the His-tagged proteins to rescue the adenine auxotrophy of this double mutant strain.

Purification of His-tagged Proteins—The pET16b-ADE16 construct and the pREP4-GroESL plasmid (10) were co-transformed into E. coli BL21(DE3) cells (Novagen, Madison, WI). Transformants containing both plasmids were used to inoculate 1 liter of 2YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) containing 50 μg/ml ampicillin and 30 μg/ml kanamycin. Cells were grown at 37 °C to an absorbance (A₆₀₀) of 0.6 before addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1.0 mM. The culture was transferred to a 30 °C shaking incubator and allowed to grow for 3 h. Cells were harvested by centrifugation at 7000 × g for 5 min at 4 °C and then resuspended in sonication buffer (50 mM sodium phosphate, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μM β-mercaptoethanol, pH 7.8) and lysed by sonication. After centrifugation at 11,000 × g for 20 min, the crude extract was applied to a 10-mL Ni²⁺-nitrilotriacetic acid agarose column (Qiagen) equilibrated with sonication buffer, at a flow rate of 0.5 ml/ min. The column was washed with wash buffer (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 6.0) until the A₆₀₀ of the wash fraction was less than 0.01. His-tagged Ade16p was then eluted from the column with wash buffer containing a 0.0–0.5 M imidazole gradient. Elution fractions were collected and assayed for AICAR transformylase activity. A stirred cell apparatus was used for protein concentration and for buffer exchange (into 50 mM sodium phosphate, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, pH 7.8).

The pET16b-ADE17 construct was transformed into E. coli BL21(DE3) cells for expression and purification of Ade17p. A 1-liter culture of BL21(DE3)/pET16b-ADE17 in 2YT/ampicillin was grown at 37 °C to an absorbance (A₆₀₀) of 0.6 before addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1.0 mM. The cells were grown in a shaking incubator at 37 °C for 3 h. After induction, cells were harvested by centrifugation at 7000 × g for 5 min at 4 °C. Purification and concentration of Ade17p was carried out in the same manner as described above for Ade16p.

Enzyme Assays—AICAR transformylase activity of the purified proteins was assayed spectrophotometrically by monitoring the formation of THF at A₂₈₂₀ as described by Black et al. (11). The assays were performed in an HP8542A diode array spectrophotometer. The reaction mixture contained a final concentration of 33 mM Tris-Cl (pH 7.4), 25 mM KCl, 5 mM β-mercaptoethanol, 0.1 mM 10-formyl-THF, and 0.05 mM AICAR in a final volume of 500 μl. The concentration of THF formed was calculated using the difference between the extinction coefficients of 10-formyl-THF and THF at 298 nm, 19.7 × 10³ M⁻¹ cm⁻¹ (11).

A thin layer chromatography radioassay (1) was used to detect AICAR transformylase activity in yeast mitochondrial and cytosolic extracts (15–30 μg of protein/assay). The assay measures the incorporation of [³⁵S]formate into [¹⁴C]formyltetrahydrofolate, [³⁵S]FAICAR, and ultimately [³⁵S]IMP.

IMP transformylase activity of the purified proteins was assayed by monitoring the formation of IMP at A₂₈₀ according to the method of Rayl et al. (12): FAICAR was a gift from G. P. Beardsley laboratory (Yale University). The reaction mixture contained 100 mM Tris-Cl (pH 7.4) and 0.1 mM FAICAR. After blanking the spectrophotometer, the enzyme was added to a final reaction volume of 500 μl. The concentration of IMP formed was determined using the difference between the extinction coefficients for IMP and FAICAR at A₂₈₀. 5.71 × 10⁵ M⁻¹ cm⁻¹ (13).

Kinetic Analysis—Steady state kinetic parameters for the purified enzymes were determined using the standard AICAR transformylase spectrophotometric assay. 7.5 μg of purified His-tagged protein was used for each reaction. The concentration of AICAR was varied from 1 to 130 μM at three different 10-formyl-THF concentrations. Similarly, the concentration of 10-formyl-THF was varied from 5 to 180 μM at three different AICAR concentrations. Both substrate series were performed in triplicate. Substrate-velocity data were plotted and fit to the Michaelis-Menton equation using nonlinear regression with Deltagraph Pro3 software on a Macintosh computer. In order to obtain Kₘ values for either AICAR or 10-formyl-THF with completely saturating conditions for the other substrate, slope and intercept replots of "apparent" kinetic parameters were done. The initial estimates for Kₘ and Vₘₐₓ values were determined directly from nonlinear regression analysis. Replots of the apparent Kₘ/Vₘₐₓ versus 1/Substrate (slope replot) and 1/Vₘₐₓ versus 1/Substrate (intercept replot) were used to determine actual kinetic parameters by extrapolation to infinite substrate concentration (14).

Chemical Cross-linking of Ade16p and Ade17p—The subunit arrangement of Ade16p and Ade17p was investigated by chemically cross-linking the proteins with dimethyl suberimidate (15). For cross-linking of Ade16p, 50 or 140 μg/ml protein was incubated in a reaction mixture containing 0.02 mM KC1, 0.2 mM triethanolamine, and 2.5 mg/ml dimethyl suberimidate (pH 8.5). The reactions were incubated for 3 h at room temperature in a final volume of 50 μl. Cross-linking of Ade17p was performed in the same way using 60 or 140 μg/ml protein. Ten microliters of each reaction mixture were analyzed on continuous 7% SDS-polyacrylamide gels.

HIS4 Gene Replacements—The yeast H3-3 plasmid, containing a functional HIS4 gene in the Yep24 vector, was obtained from Gerald Fink (Whitehead Institute for Biomedical Research). Plasmid H3-3 was introduced into the ATY1, ATY2, and ATY3 yeast strains using a lithium acetate transformation procedure. Uracil-protoprototrophic transformants were selected for the ability to rescue the histidine requirements of these ade16Δ and ade17Δ mutant yeast strains. Gene replacement (16) was used to replace the disrupted his4Δ gene in these strains with a wild-type HIS4 gene. HIS4Δ gene replacements were carried out using oligonucleotide primers, HIS4AS and HIS4AS, to amplify the HIS4 gene from X2180 yeast genomic DNA. The amplified DNA was purified using the Qiagen PCR purification kit (Qiagen) and used to transform ATY1 and ATY3 yeast strains using a lithium acetate transformation procedure. Uracil-protoprototrophic transformants were selected for the ability to rescue the histidine requirements of these ade16Δ and ade17Δ mutant yeast strains. Gene replacement was verified by PCR using the same HIS4 oligonucleotides. A ade16Δ ade17Δ HIS4Δ yeast strain was constructed by disrupting the ATY1 strain with an ade17Δ::URA3 construct and selecting for uracil prototrophs. The resulting yeast strain was named ATY3.1 (Table I).

Subcellular Fractionation—Cytosolic and mitochondrial fractions (extracts) were isolated from DAY4 yeast using the subcellular fractionation method of Daum et al. (17), except that bovine serum albumin was eliminated in all buffers. Cells were grown in lactic acid media to increase mitochondrial yield. Isolated mitochondria were resuspended in sodium phosphate lysis buffer (10 mM NaH₂PO₄, pH 7.4, 1 mM EDTA, 1 mM β-mercaptoethanol, 10 mM phenylmethylsulfonyl fluoride) and disrupted by vortexing with glass beads. Isolated fractions were assayed for AICAR transformylase activity using the thin layer chromatography radioassay described previously (1). The NAD-dependent 5,10-methylene tetrahydrofolate dehydrogenase activity was followed as a cytosolic marker, using a previously described assay (18). The NAD-dependent isocitrate dehydrogenase activity was assayed as a mitochondrial marker, using the method of Keys and McAlister-Henn (19).

Epitope Tagging—Centromeric and integrating vectors containing c-myc-tagged ADE16 or c-myc-tagged ADE17 were constructed. Epitope-tagged ADE16 was constructed in the pRS416 centromeric vector (20) by the "insertional mutation by overlap extension" method (21). Oligonucleotides ADE16Xho and ADE16OBe were used to amplify a PCR product containing sequence upstream of the ADE16 translation junction.
Yeast AICAR Transformylase Isozymes

TABLE I  
Yeast strains

| Strain | Genotype | Reference |
|--------|----------|-----------|
| DAY4   | a ura3–52 trp1 leu2 his4 ser1 | 18 |
| X2180  | SUC2 mal1 gal2 CUP1 | Yeast Genetic Stock Center |
| ATY1   | a ura3–52 trp1 leu2 his4 ser1 Δade17 | 1 |
| ATY2   | a ura3–52 trp1 leu2 his4 ser1 Δade16 | 1 |
| ATY3   | a ura3–52 trp1 leu2 his4 ser1 Δade16 Δade17 | 1 |
| ATY1.1 | a ura3–52 trp1 leu2 ser1 Δade16 HIS4 | This study |
| ATY2.1 | a ura3–52 trp1 leu2 ser1 Δade17 HIS4 | This study |
| ATY3.1 | a ura3–52 trp1 leu2 ser1 Δade16 Δade17 HIS4 | This study |
| ATY4   | a ura3–52 trp1 leu2 ser1 Δade16 c-mycADE17 HIS4 | This study |
| ATY5   | a ura3–52 trp1 leu2 ser1 Δade16 c-mycADE17 HIS4 | This study |

TABLE II  
Sequences of PCR primers

| Primer            | Sequence |
|-------------------|----------|
| NdeATIC1          | GACCCGATAATGCGAAATATACCC |
| BamATIC1          | CATATCTACTATGGTTG |
| NdeATIC2          | GAGGCCATATGGCGCACTACAC |
| BamATIC2          | GTACGATCCATCTAATGGTG |
| HIS4S             | TGCACCAGTCGATACACG |
| HIS4AS            | CCACCTCTGTGTAATACCTG |
| ADE16Xho          | TTATTCGGAGATCCCTCCTCACAGCCAGAC |
| ADE16OE           | CTTCCAGAATAATGTTTTTGTCCCTATTGATCGCTTTTGTGTTTGGTC |
| epi16S            | GAACAAAAACATCTTTGCGGAGATGTCGCGCGCAATATAACCAGACGTG |
| ADE16Xba          | CGCCCTTCAGATTCGGGCAACATTTGTTG |
| epi17S            | GAACAAAAACATCTTTGCGGAGATGTCGCGCGCAATATAACCAGACGTG |
| epi17AS           | CATATCTGAGGTGAATGTTG |
| ADE17Xho          | TTATTCGGAGATCCCTCCTCACAGCCAGAC |
| ADE17OE           | CTTCCAGAATAATGTTTTTGTCCCTATTGATCGCTTTTGTGTTTGGTC |
| ADE17St            | TACGAGCTCTAATACATGTGTTGTC |
| c-mycS            | CGATATCTGTCAGGAGGACGCG |
| c-mycAS           | CTATTATCTGCGGAGAGACGCG |
| ppADE16           | TATAGACCTAGGCTGTCAT |

Preparation of Yeast Extracts for Gel Electrophoresis and Immunoblotting—Yeast cultures were grown in minimal media to late log phase and harvested. Crude extracts were prepared by suspending washed cells in 25 mM Tris-CI (pH 7.6), 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, disrupting with glass beads, and centrifugation at 25,000 × g for 30 min. Protein concentrations of the supernatants were
determined using the Bradford protein assay with bovine serum albumin as a standard. Extracts were resolved on 10% polyacrylamide gels, and proteins were transferred to nitrocellulose membranes by electroblotting. Immunoblotting was performed using anti-c-myc mouse monoclonal antibodies (9E10; Santa Cruz Biotechnology, Inc.) diluted to 0.25 μg/ml (10 mM Tris-HCl (pH 8.0), 150 mM NaCl) with 1% dry milk and 1% normal goat serum (Jackson ImmunoResearch). Horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Roche Molecular Biochemicals) were diluted 1:10,000 in TBS with 0.05% Tween-20. Tagged proteins were visualized using an ECL (enhanced chemiluminescence) detection system (Amersham Pharmacia Biotech). Autoradiograms were developed manually and then scanned to generate a digital image. Quantification of band intensities was performed using Kodak Digital Science 1D Image Analysis Software.

**RESULTS**

**Expression of Yeast Ade16p and Ade17p**—The pVT-HisADE16 and pVT-HisADE17 vectors were transformed separately into the ATY3.1 (Δade16 Δade17) adenine auxotroph. Both plasmids rescued the adenine auxotrophy of this strain, indicating that His-tagged versions of Ade16p and Ade17p are functional and support purine biosynthesis in vivo. We therefore proceeded to purify these His-tagged proteins for kinetic characterization.

His-tagged Ade16p and Ade17p were expressed in *E. coli* from the pET vectors, pET16b-ADE16 and pET16b-ADE17. Expression of soluble Ade16p required coexpression of the *E. coli* GroEL and GroES proteins from the pREP4-GroEL-GroES plasmid (10), along with a shift in induction temperature from 37°C to 30°C. The pREP4-GroEL-GroES plasmid, a gift from Dr. Martin Stieger (Hoffman-La Roche), contains the *E. coli* genes encoding GroEL and GroES behind the Lac promoter/operator element. This construct allowed for overproduction of these chaperonins in conjunction with overexpression of Ade16p. Without coexpression of the chaperone proteins, all Ade16p protein was found to be in the insoluble particulate fraction of the cells. Coexpression of GroES and GroEL allowed approximately 50% of Ade16p to remain in the soluble fraction. This was sufficient to allow purification by Ni²⁺-chelate chromatography. His-tagged Ade17p expressed from pET16b-ADE17 in *E. coli* remained in the soluble fraction of the cells and did not require coexpression of GroEL and GroES.

**Purification and Kinetic Characterization of Ade16p and Ade17p**—His-tagged Ade16p and Ade17p were purified from *E. coli* soluble extracts to homogeneity in one step using Ni²⁺-nitrilotriacetic acid agarose column chromatography. Both Ade16p and Ade17p are represented by single proteins with apparent molecular masses of 66,000 daltons, as determined by denaturing gel electrophoresis (Fig. 2). Ade16p exhibited specific AICAR transformylase activity of 1.0 μmol of THF formed/min/mg, and Ade17p had a specific activity of 0.9 μmol of THF formed/min/mg. Ade16p and Ade17p were also shown to be bifunctional, catalyzing the IMP cyclohydrolase reaction as well. This activity was followed spectrophotometrically by monitoring the formation of IMP at A_{248}. IMP cyclohydrolase activity was detected in both purified yeast enzymes, with Ade16p having a specific IMP cyclohydrolase activity of 2.0 ± 0.5 μmol of IMP formed/min/mg and Ade17p having a specific activity of 1.7 ± 0.4 μmol of IMP formed/min/mg. The steady state kinetic parameters for the AICAR transformylase activity of the purified enzymes were determined using slope and intercept plots and extrapolating to infinite substrate concentration. The AICAR K_m values for the two yeast enzymes were essentially identical, whereas the 10-formyl-THF K_m for Ade16p was twice that observed for Ade17p. The low micromolar K_m values exhibited by the yeast enzymes are quite similar to those reported for purified human AICAR transformylase/IMP cyclohydrolase (12). These results are summarized in Table III.

To investigate the subunit arrangement of Ade16p and Ade17p, the purified proteins were incubated at two different concentrations with the chemical cross-linker dimethyl suberimidate and analyzed using continuous SDS-PAGE. Both Ade16p and Ade17p form dimers under these conditions, as indicated by the presence of a protein band approximately twice the size of the individual Ade16p or Ade17p monomers (Fig. 3). No evidence of higher oligomers was seen. The human enzyme has been shown by equilibrium sedimentation experiments to exist in a monomer/dimer equilibrium with a K_d of 0.55 μM (26).

**Histidine Auxotrophy of ade16 ade17-disrupted Yeast**—The purine and histidine biosynthesis pathways are interconnected by the intermediate AICAR, which is a by-product of the sixth step of histidine biosynthesis and is the substrate for the AICAR transformylase reaction. The first genetic evidence for cross-talk between these two pathways came when *ade3* yeast mutants were found to require histidine in addition to adenine (27). We wondered whether AICAR transformylase mutants also required histidine. However, the original *ade16* and *ade17* disrupted yeast strains (ATY1, ATY2, and ATY3) were constructed in a his4 mutant background (1). When ATY1 and ATY2 were transformed with a plasmid-borne HIS4 gene, the strains became prototrophic for histidine. However, the HIS4 plasmid did not rescue the histidine auxotrophy of the double mutant strain, ATY3. This result was confirmed when we introduced HIS4 in single copy by gene replacement of the chromosomal *his4* loci of the strains. ATY1.1 and ATY2.1 strains were able to grow at wild-type rates without histidine, whereas the ATY3.1 strain (Δade16 Δade17 Δhis4) required the addition of both adenine and histidine to the media for growth (Fig. 4). The *ade16 ade17* double disruptant strain is the only *de novo* purine biosynthesis mutant, other than *ade3*, that is auxotrophic for both adenine and histidine.

c-myc-tagged Ade16p and Ade17p Are Functional in Vivo—The centromeric plasmids (pRS416-c-mycADE16, pRS416-c-mycADE17) and the integrating plasmids (pRS306-c-mycADE16 and pRS306-c-mycADE17) were generated for the
Localization of Ade16p and Ade17p—Analysis of the amino acid sequences of Ade16p and Ade17p by PSORT suggested a cytoplasmic localization for both proteins. There was no evidence of a mitochondrial presequence, a nuclear localization signal, or any other targeting signal in either sequence. To test this prediction, subcellular fractionation of DAY4.1 (ADE16 ADE17), ATY1.1 (ade16 ADE17), and ATY2.1 (ADE16 ADE17) was carried out. NAD-dependent 5,10-methylenetetrahydrofolate dehydrogenase activity was assayed as a cytosolic marker protein, and NAD-dependent isocitrate dehydrogenase was used as a mitochondrial marker enzyme. The cytosolic and mitochondrial fractions were assayed for AICAR transformylase activity using a sensitive thin layer chromatography AICAR transformylase assay (1). The cytosolic fraction of the wild-type strain exhibited an activity of 0.85 nmol of IMP formed/min/µg, whereas activity in the mitochondrial fraction was undetectable (data not shown). Chromatograms for the disruption strains gave cytosolic activities of 0.51 and 0.05 nmol of IMP formed/min/µg for ATY1.1 and ATY2.1, respectively. Again, activity in the mitochondrial fractions of these strains was undetectable. Thus, both Ade16p and Ade17p appear to be active only in the cytoplasm of yeast, with Ade16p accounting for only ~10% of the AICAR transformylase activity, as previously observed (1).

Immunofluorescent labeling confirmed the cytoplasmic localization of Ade16p and Ade17p. Yeast strains ATY4 and ATY5, expressing c-mycAde16p and c-mycAde17p, respectively, were labeled using anti-c-myc antibodies. Immunofluorescent staining was also performed with DAY4.1 yeast (containing no c-myc-tagged proteins) as a negative control. In ATY4 and ATY5, specific staining was seen diffusely throughout the cytosol, with no apparent subcytosolic localization of either isozyme (Fig. 5). Staining of epitope-tagged Ade16p (Fig. 5D) was much less intense than Ade17p (Fig. 5B) but still significantly above the background staining (Fig. 5F).

Cellular Levels of Ade16p and Ade17p—Western blotting of ATY4 and ATY5 yeast extracts was used to analyze the relative levels of Ade16p and Ade17p in the cell. A dilution series of ATY4 crude extracts and a similar dilution series of ATY5 extracts were resolved on the same SDS-polyacrylamide gel and subsequently transferred to a nitrocellulose membrane. The immunoblots were processed with anti-c-myc antibodies and chemiluminescence detection, and the resulting band intensities were compared. Serial dilutions were used in these experiments in order to minimize any errors in gel loading and the inherent inaccuracy of protein determination. The band intensities were plotted versus the total protein in each lane to generate linear graphs representing the expression of Ade16p and Ade17p. The slopes of the two lines were compared, and it was determined that Ade17p was approximately 4–5 times more abundant in the cell than Ade16p, when cells were grown on glucose (Fig. 6).
expression was tested using the ATY4 yeast strain. No differences were seen between the levels of Ade16p in cells grown with or without adenine (Fig. 7B).

An experiment was also performed to determine the time course of Ade17p repression. ATY5 cells were grown to an absorbance of 0.3 before addition of 20 μg/liter adenine. Cells were removed from the culture and harvested at 3, 6, 9, and 12 h after the addition of adenine. (The absorbances of these cultures ranged from 0.3 to 4.5 during this time period.) Cell extracts were prepared, and the levels of Ade17p were compared by immunoblotting (Fig. 8). Some reduction of Ade17p was seen at 3 h, but significant repression was not seen until 9 h after addition of adenine. The level of Ade17p remained fairly constant between 9 and 12 h after the addition of adenine to the media. A similar experiment was performed simultaneously to monitor Ade17p levels without the addition of adenine. Ade17p levels remained essentially constant throughout the same time course (data not shown).

The Effect of Histidine, Methionine, and 3-Aminotriazole on Ade16p and Ade17p Expression—Immunoblotting experiments were performed on extracts from ATY4 and ATY5 yeast grown with the addition of histidine, 3-aminotriazole, or methionine to the media. The effects of histidine and 3-aminotriazole were tested because of the interconnection between histidine and purine biosynthesis. Step 6 of histidine biosynthesis produces AICAR as a byproduct, which can be utilized by de novo purine biosynthesis (29). One hypothesis for the presence of two AICAR transformylase isozymes is that one of the enzymes is associated more with amino acid biosynthesis, metabolizing the AICAR made during histidine production, whereas the other enzyme is more closely associated with the enzymes involved in de novo purine metabolism. 3-Aminotriazole inhibits the seventh step of histidine biosynthesis (imidazoleglycerolphosphate dehydratase) and was used to determine whether a block in the histidine biosynthesis pathway affects the expression of Ade16p or Ade17p. Cells grown in 3-aminotriazole were not supplemented with histidine, and therefore only grew to an approximate A600 of 0.4. ATY4 and ATY5 yeast were also grown with the addition of methionine, because of the indirect links between purine and methionine synthesis via one-carbon metabolism. In all three experiments described here, there were no observable differences in the expression of Ade16p or Ade17p under these growth conditions (data not shown). The Ade16p and Ade17p levels were similar in cells grown with or without the addition of histidine, 3-aminotriazole, or methionine to the media.

Ade16p and Ade17p Expression on Nonfermentable Carbon
Sources—The expression of Ade16p and Ade17p was compared in cells grown with fermentable versus nonfermentable carbon sources. ATY4 and ATY5 cultures were grown with 2% glucose or 3% glycerol plus 2% ethanol and harvested at late log phase. Immunoblots showed that the expression of Ade16p was somewhat increased in glycerol/ethanol, whereas the level of Ade17p was slightly decreased in cells grown with this carbon source (Fig. 9). The Ade16p level appears to be 1.5 times higher in cells grown with the nonfermentable carbon source than in the cells grown with glucose. Ade17p levels did not change significantly (less than 20% difference in slopes) when grown with glycerol and ethanol. Yeast strains containing c-myc-tagged ADE16 or ADE17 were also grown using acetate as the carbon source rather than glucose. Again, Ade16p expression was increased on this nonfermentable carbon source, with acetate-grown cells having 2 times higher Ade16p expression than glycerol/ethanol-grown cells (data not shown). The expression of Ade17p was not significantly affected by growth on acetate. After observing the effects of these nonfermentable carbon sources on Ade16p expression, a similar experiment was performed using galactose, a fermentable but nonrepressing carbon source, where earlier work had not revealed any effect of adenine on the expression of Ade16p (2).

Immunoblotting experiments also demonstrated the difference between the regulation of Ade16p and Ade17p by adenine. As with other purine biosynthesis enzymes, the expression of Ade17p is repressed by adenine, whereas Ade16p expression was not affected by the addition of adenine to the media. This result was expected because the ADE16 gene does not contain a Bas1p binding site in the promoter region and because earlier work had not revealed any effect of adenine on the expression of ADE16 (2).

The expression of Ade16p was increased when cells were grown on nonfermentable carbon sources. Cells grown on acetate or glycerol plus ethanol expressed 1.5–2 times more Ade16p than glucose-grown cells. This small increase in Ade16p expression on nonfermentable carbon sources was an unexpected result, for which there are no clear explanations at this time. The increase in Ade16p expression is probably not a result of glucose derepression, because Ade16p levels were not affected by growth on galactose (as compared with glucose). Meiosis and spore formation are cellular processes that are controlled by the levels of certain carbon sources. Meiosis is blocked by the presence of glucose, but it is dependent on a nonfermentable carbon source, such as acetate (30). It is possible that Ade16p is the preferred AICAR transformylase isozyme when cells are lacking glucose and are forced to either
sporulate or use an alternate route to meet their energy requirements.

A second possibility is that the increase in Ade16p levels in yeast strains with ade16 or ade17 disruptions in combination with mutations of other folate-metabolizing enzymes.

Another hypothesis explaining the presence of two AICAR transformylase isozymes in S. cerevisiae is that one enzyme may be more involved with utilizing the AICAR from histidine biosynthesis, whereas the other is involved in the metabolism of AICAR from the de novo purine pathway. In particular, we speculated that Ade16p might be responsible for metabolizing the AICAR produced by the histidine synthesis pathway, and the of metabolizing the AICAR produced by the histidine synthesis pathway, of its low, constitutive expression even in the presence of adenine.

We found no evidence for this in the experiments presented here, however, because the addition of histidine did not affect the expression of either isozyme and because 3-amino-1,2,4-triazole, which inhibits histidine biosynthesis, did not affect the regulation of Ade16p or Ade17p. This repression occurs at the level of transcription as reported by Daignan-Fornier and involves the transcription factors Bas1p and Bas2p (2, 34).

In summary, these results show that the enzymes encoded by ADE16 and ADE17 are co-regulated with purine biosynthesis genes, whereas genes encoding steps downstream of AICAR production are not regulated. However, when yeast are grown with adenine in the media in the ability to formylate AICAR would accumulate this intermediate, which in turn might inhibit histidine biosynthesis in a feedback or other regulatory manner, thus leading to a histidine requirement. This question deserves further study and will require a determination of the relative contributions of the histidine pathway and the purine pathway to the overall production or accumulation of AICAR.

In summary, these results show that the enzymes encoded by ADE16 and ADE17 are functionally indistinguishable with respect to certain activity, cellular localization, and subunit organization. They differ significantly in their expression levels and patterns. In cells growing under normal conditions, Ade17p is the predominant isozyme and appears to be a classic adenine-responsive enzyme, being strongly repressed by adenine. This repression occurs at the level of transcription as reported by Daignan-Fornier and involves the transcription factors Bas1p and Bas2p (2, 34). Ade16p, the minor isozyme, appears to be expressed constitutively, unresponsive to adenine levels. However, Ade16p shows a small response to non fermentable carbon sources, perhaps suggesting a role for this isozyme in respiration or sporulation. Only a limited number of conditions were tested here, and the complete metabolic role of Ade16p remains to be elucidated.

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