An NADH-dependent L-xylulose reductase and the corresponding gene were identified from the yeast *Ambrosiozyma monospora*. The enzyme is part of the yeast pathway for L-arabinose catabolism. A fungal pathway for L-arabinose utilization has been described previously for molds. In this pathway L-arabinose is sequentially converted to L-arabinitol, L-xylulose, xyitol, and D-xylulose and enters the pentose phosphate pathway as D-xylulose 5-phosphate. In molds the reductions are NADPH-linked, and the oxidations are NAD⁺-linked. Here we show that in *A. monospora* the pathway is similar, i.e. it has the same two reduction and two oxidation reactions, but the reduction by L-xylulose reductase is not performed by a strictly NADPH-dependent enzyme as in molds but by a strictly NADH-dependent enzyme. The *ALX1* gene encoding the NADH-dependent L-xylulose reductase is strongly expressed during growth on L-arabinose as shown by Northern analysis. The gene was functionally overexpressed in *Saccharomyces cerevisiae* and the purified His-tagged protein characterized. The reversible enzyme converts L-xylulose to xyitol. It also converts D-ribulose to D-arabinitol but has no activity with L-arabinitol or adonitol, i.e. it is specific for sugar alcohols where, in a Fischer projection, the hydroxyl group of the C-2 is in the L-configuration and the hydroxyl group of C-3 is in the D-configuration. It also has no activity with C-6 sugars or sugar alcohols. The *Kₘ* values for L-xylulose and D-ribulose are 9.6 and 4.7 mM, respectively. To our knowledge this is the first report of an NADH-linked L-xylulose reductase.

L-Arabinose is a major constituent of plant material so that L-arabinose catabolism is relevant for microorganisms living on decaying plant material. For the catabolism of L-arabinose two distinctly different pathways are known, a bacterial pathway and a fungal pathway. In the bacterial pathway the three enzymes L-arabinose isomerase, L-ribulokinase, and L-ribulose-5-phosphate 4-epimerase convert L-arabinose to D-xylulose 5-phosphate. A fungal pathway was described for molds but not for yeasts. It was first described by Chiang and Knight (1) for the mold *Penicillium chrysogenum*, in which L-arabinose is converted to D-xylulose 5-phosphate, as in bacteria, but through the enzymes aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, xyitol dehydrogenase, and xylulokinase. In this pathway the aldose reductase and the L-xylulose reductase use NADPH as cofactor, whereas L-arabinitol 4-dehydrogenase and xyitol dehydrogenase use NAD⁺ as cofactor (Fig. 1). The same pathway was described for the mold *Aspergillus niger* (2). The pathway was expressed in the yeast *Saccharomyces cerevisiae* using genes from the mold *Hypocrea jecorina* (*Trichoderma reesei*) and other fungal sources and shown to be functional, i.e. the resulting strain could grow on and ferment L-arabinose, however, at very low rates (3–5).

Information about the corresponding pathway in yeast is rare. Shi et al. (6) provided evidence that the yeast pathway requires a xyitol dehydrogenase. In a mutant of *Pichia stipitis*, which was unable to grow on L-arabinose, overexpression of a xyitol dehydrogenase could restore growth on L-arabinose. Dien et al. (7) tested more than 100 yeast species for L-arabinose fermentation. Most of them produced arabinitol and xyitol indicating that the yeast pathway is similar to the pathway of molds and not to the bacterial pathway. However, there is only limited knowledge about the enzymes of this pathway in yeast. Aldose reductases, which are active with L-arabinose and D-xylose, were described for the yeasts *S. cerevisiae* (8) and *P. stipitis*, for example (9). The enzymes have similar affinity toward D-xylose and L-arabinose and convert both sugars with a similar rate. The *S. cerevisiae* enzyme, however, is strictly NADPH-dependent, whereas the *P. stipitis* enzyme can use both NADH and NADPH but has a preference for NADPH. For the yeast *Candida parapsilosis* an aldose reductase with a preference for NADH was described (10). There are no reports about L-arabinitol-4-dehydrogenases or L-xylulose reductases in yeast. Xyitol dehydrogenase and xylulokinase were characterized in *S. cerevisiae* (11, 12) and *P. stipitis* (13, 14). The xyitol dehydrogenases are strictly NAD⁺-linked similar to the enzymes in mold.

L-Xylulose reductases are not only described for molds but also for higher animals. The activity was found in guinea pig liver (15). A partly purified protein from pigeon liver is commercially available (Sigma). From hamster liver a gene was identified, which coded for a diacetyl reductase that had also L-xylulose reductase activity (16). All these L-xylulose reductase activities have in common that they are strictly coupled to NADPH. To our knowledge there is no report about L-xylulose reductase activity coupled to NADH.

**EXPERIMENTAL PROCEDURES**

**Strains**—The *Escherichia coli* strain DH5α was used in the cloning procedures. The *Ambrosiozyma monospora* strain NRRL Y-1484 was used for cDNA synthesis and Northern blot analysis. The *S. cerevisiae* strain H2651 (4) was used in the screening for improved growth on L-arabinose. The H2651 has the *P. stipitis* *XYL1* and *XYL2* genes encoding xylose reductase and xyitol dehydrogenase, respectively, ex-
was cultured in Yeast Nitrogen Base (Difco) with 4% L-arabinose as the tryptophan when the strains were carrying plasmids. Synthetic complete (SC) medium (18) lacking uracil, leucine, and/or marker genes. Carried on separate multicopy expression vectors with the URA3 gene coding for the NADH-dependent L-xylulose reductase was transferred respectively, integrated into the URA3 locus. The endogenous XKS1 gene encoding xylulokinase under the modified ADH1 promoter is integrated into the HIS3 locus. The lxd1 and lxr1 genes coding for L-arabinitol dehydrogenase and L-xylulose reductase from H. jecorina (T. reesei) are carried on separate multi-copy expression vectors with LEU2 and URA3 marker genes.

The S. cerevisiae strain H1346 (CEN.PK2) was used to produce the histidine-tagged enzyme. All S. cerevisiae strains were cultured in synthetic complete (SC) medium (18) lacking uracil, leucine, and/or tryptophan when the strains were carrying plasmids.

**Construction of the cDNA Library—A. monospora** (NRRL Y-1484) was cultured in Yeast Nitrogen Base (Difco) with 4% L-arabinose as the carbon source. The cells were grown overnight at 30 °C and harvested by centrifugation when the L-arabinose concentration was about 2%. Total RNA was extracted from the cells with the Trizol reagent kit (Invitrogen) according to the manufacturer’s instructions. The mRNA was isolated from the total RNA with Oligotex mRNA mini kit (Qiagen). The cDNA was synthesized by the SuperScript cDNA synthesis kit (Invitrogen), and the fractions containing cDNA were pooled and ligated into the Sall-NotI cut pEXP-AD502 vector (Invitrogen). This plasmid is a centromeric expression vector that has the ADH1 promoter and N-terminal GAL4 fusion fragment in front of the insert. The ligation mixture was transformed into the E. coli DH5α strain by electroporation following the manufacturer's (Bio-Rad) instructions. After overnight incubation about 100,000 independent colonies were pooled from ampicillin plates and stored in –80 °C in 50% glycerol + 0.9% NaCl. Before extracting plasmids from the transformants, the library was amplified by growing it in LB medium for 4 h of shaking at 37 °C.

**Screening the cDNA Library for Growth on L-Arabinose**—The S. cerevisiae strain H2651 was transformed with the cDNA library using the Gietz Lab transformation kit (Molecular Research Reagents). The plasmids contained the ADH1 promoter and the ALX1 gene expression vector with the Trizol reagent kit (Invitrogen). 4 mM lysozyme was added to the N terminus of the protein by amplifying the A. monospora i.e. 

**Northern Blot Analysis—A. monospora** (NRRL Y-1484) was cultured overnight in 50 ml of YNB medium with 2% d-fructose, glycerol, d-glucose, L-arabinose, or d-xylose as the carbon source. An S. cerevisiae strain carrying the ALX1 gene expression vector and an S. cerevisiae strain with an empty vector were cultured overnight in SC-ura + 2% glucose medium. All cultures were done in duplicate. The cells were harvested by centrifugation, and total RNA was extracted with the Trizol reagent kit (Invitrogen). 4 μg of the total RNA was used as sample in the gel electrophoresis. The RNA amount loaded on the gel was checked by staining (Fig. 4, lower panel) with the SYBR Green II RNA gel stain (BMA Biomedicals). The Northern blot analysis was carried out using standard procedures. The probe for the Northern blot analysis was an 800-bp fragment that was obtained with EcoRV-BamHI digestion from a TOPO vector containing the His-tagged construct. The digestion releases the tag from the probe. The probe was labeled with [α-32P]dCTP (Amersham Biosciences) using a commercial kit for DNA labeling (Roche Applied Science).

### RESULTS

**Screening and Identification of the L-Xylulose Reductase**—

We constructed a cDNA library from L-arabinose grown A. monospora, one of the rare yeast species able to ferment L-arabinose to ethanol. In order to clone genes that would be involved or would enhance L-arabinose utilization, the cDNA library was transformed to a recombinant S. cerevisiae strain that already contained the L-arabinose utilization pathway, i.e.

| Enzyme Pathway | Bacterial Pathway |
|----------------|------------------|
| L-arabinose reductase | L-arabinose dehydrogenase |
| L-arabinitol reductase | L-arabinitol dehydrogenase |
| D-xylulose reductase | L-xylulose reductase |
| xylitol dehydrogenase | D-xylulose dehydrogenase |
| xylulokinase | D-xylulose kinase |

**Fig. 1. The microbial pathways for L-arabinose catabolism.** For the catabolism of L-arabinose, bacterial and fungal pathways are described. In both pathways L-arabinose is converted to D-xylulose 5-phosphate. A fungal pathway is described for molds. Here the L-arabinose goes through two NADPH-linked reductions and two NADH-linked oxidations. The bacterial pathway is free of reduct reactions.
the genes for aldose reductase, xylitol dehydrogenase, and xylylokinase and also mold (H. je corina) genes for L-arabinose dehydrogenase and L-xylulose reductase (see “Experimental Procedures”). Although this strain can utilize L-arabinose, it grows so slowly on L-arabinose that we were able to select for improved growth on this carbon source. The transformants were first plated on glucose plates and then replicated on plates that had L-arabinose as the sole carbon source. After about 2 weeks of cultivation, colonies were picked and plasmids extracted from them. One of the clones contained a plasmid that carried an open reading frame coding for a 272-amino acid product with a molecular mass of 29,495 Da. The open reading frame coded for a protein of the family of short chain alcohol dehydrogenases with high amino acid similarity to d-arabinitol dehydrogenases found from P. stipitis, Candida albicans, and Candida tropicalis. In addition it had lower similarities to the lxr1 gene of H. je corina that codes for l-xylulose reductase. The gene was named ALX1 for A. monospora l-xylulose reductase. The sequence was submitted into the GenBank™ and has the accession number AJ583159.

Overexpression in S. cerevisiae and Purification of the Histidine-tagged L-Xylulose Reductase—The open reading frame of the ALX1 gene was ligated to a yeast expression vector with the strong PGK1 promoter. The plasmid was then transformed to a yeast strain where no other enzymes of the L-arabinose pathway were expressed. In the crude yeast extract we found enzyme activity with xylitol (about 31 nanokatals/mg) and d-arabinitol (about 30 nanokatals/mg) when NAD⁺ was used as a cofactor, but no activity was observed with NADP⁺ as cofactor. The NAD⁺-linked xylitol and d-arabinitol dehydrogenase activities were absent in the control strain lacking the ALX1 gene.

For a more detailed analysis of the kinetic properties, we used a purified enzyme. To facilitate the purification a histidine tag was added. Because our previous studies showed that a C-terminal histidine tag in an enzyme belonging to the short chain alcohol dehydrogenase family may lead to an inactive enzyme (4), we added the histidine tag to the N terminus of the protein. The activity of the histidine-tagged protein was confirmed by measuring the activity from yeast cell lysates. The activity levels were similar in the strains expressing the histidine-tagged or unmodified protein, about 85 nanokatals/mg with xylitol and 87 nanokatals/mg with d-arabinitol. The open reading frame coded for a protein of the family of chain alcohol dehydrogenase family may lead to an inactive enzyme (4), we added the histidine tag to the N terminus of the enzyme (4), we added the histidine tag to the N terminus of the protein. The activity of the histidine-tagged protein was confirmed by measuring the activity from yeast cell lysates. The activity levels were similar in the strains expressing the histidine-tagged or unmodified protein, about 85 nanokatals/mg with xylitol and 87 nanokatals/mg with d-arabinitol. Fig. 2 shows the SDS-PAGE of the His-tagged protein, eluted from the nickel-nitrilotriacetic acid column. The molecular mass was about 32 kDa.

Kinetic Characterization—The purified His-tagged protein was used to determine the kinetic parameters of the enzyme. The Michaelis-Menten constants were derived from an Eadie-Hofstee plot. All measurements were done at least in triplicate. The Kₘ for d-ribulose was 4.7 ± 0.8 mM and the Kₘ for l-xylulose was 9.6 ± 0.7 mM. The Vₘₐₓ values were 2700 ± 300 nanokatals/mg for d-ribulose and 1700 ± 300 nanokatals/mg for l-xylulose. The kinetic parameters of the reverse reactions were Kₘ 2.7 ± 0.3 mM and Vₘₐₓ 600 ± 50 nanokatals/mg for d-arabinitol and Kₘ 7.2 ± 1 and Vₘₐₓ 630 ± 50 nanokatals/mg for xylitol. The results are summarized in the Fig. 3.

Substrate Specificities—Activity was observed with the sugar alcohols xylitol and d-arabinitol by using as cofactor NAD⁺. No activity was observed with the C-5 sugar alcohols l-arabinitol and adonitol (ribitol) and the C-6 sugar alcohols dulcitol (galactitol), d-mannitol, and d-sorbitol. No activity with xylitol or d-arabinitol was found when NAD⁺ was replaced by NADP⁺.

By using sugar and NADH as substrates (forward reaction), activity was observed with l-xylulose and d-ribulose. An ~100-fold lower activity was observed with the other pentulose sugar d-xylulose. No activity was seen with the hexulose sugars d-sorbose, l-sorbose, d-psicose, and d-fructose.

Product Identification by HPLC—The largest activities were observed with d-ribulose and l-xylulose in the reducing reaction and with xylitol and d-arabinitol in the oxidizing reaction. To identify the reaction products we used HPLC. From l-xylulose xylitol was formed, but not arabinitol or adonitol (ribitol). From d-ribulose and from l-xylulose arabinitol was formed but not adonitol (ribitol) or xylitol. The HPLC method that was used does not distinguish between L- and D-arabinitol. In the reverse direction xylulose was formed from xylitol. In a reaction with d-arabinitol as the substrate both ribulose and xylulose were produced. Also here the method does not distinguish between the L and D forms.

Northern Analysis—To study the role of the l-xylulose reductase in A. monospora, the expression of the gene on different carbon sources was studied by Northern analysis. A. monospora was grown on the carbon sources d-glucose, glycerol, d-fructose, L-arabinose, and L-xylulose. The results are shown in the Fig. 4. The gene was expressed on all carbon sources that were studied. However, the strongest expression of the ALX1 gene was seen on L-arabinose. The size of the messenger RNA is ~1250 bp. As controls S. cerevisiae strains were used, one strain having the ALX1 in a multicopy expression vector and the other strain carrying an empty vector.

DISCUSSION

As described in the Introduction there are indications that yeasts and molds share the same fungal pathway for L-arabinose catabolism. A. monospora NRRL Y-1484 was identified as one of the best L-arabinose fermenting yeast strains according to Dien et al. (7). We have screened an A. monospora cDNA library in a yeast expression vector in a recombinant S. cerevi-
siae strain for improved growth of L-arabinose. The recombinant S. cerevisiae strain contained all the genes of a fungal L-arabinose pathway, i.e. the XYL1 and XYL2 from the yeast P. stipitis coding for a D-xylose reductase with L-arabinose reductase activity and xylitol dehydrogenase, respectively, the lad1 and lxr1 from the mold H. jecorina coding for L-arabinitol 4-dehydrogenase and L-xylulose reductase, respectively, and the XKS1 from S. cerevisiae coding for xylulokinase. This strain is able to grow on L-arabinose, but slowly. In the screen we found transformants with improved growth. Some of them contained an A. monospora gene encoding a protein from the short chain alcohol dehydrogenase family. Compared with other proteins in public data bases, it had highest similarity to the D-arabinitol dehydrogenases of the yeasts C. albicans (21), C. tropicalis (22), and P. stipitis (23). It had also similarity, however, to a lower degree to the L-xylulose reductase of the mold H. jecorina (4).

The purified His-tagged protein was an L-xylulose reductase that is specific for NADH. No activity was observed with NADP+/H+/NADPH, i.e. it is distinctly different from the L-xylulose reductase of H. jecorina and all other known L-xylulose reductases, which are all specific for NADP+/NADPH. The H. jecorina enzyme also catalyzed the reaction from D-mannitol to D-fructose. This activity was not observed with the A. monospora enzyme.

The enzyme reversibly converts L-xylulose to xylitol and D-ribulose to D-arabinitol (Fig. 5). It has a much lower activity with D-xylulose. This means that the enzyme has a preference for 2-ketose sugars where the hydroxyl group of the C-3 is in the D-configuration. Sugar alcohols can serve as a substrate when the hydroxyl group of C-2 is in the L- and the hydroxyl group of the C-3 is in the D-configuration in any of the possible Fischer projections. The enzyme does not have activity with sugar alcohols that do not have such a configuration, such as adonitol (ribitol) and L-arabinose. The enzyme seemed to be specific for sugar alcohols that have not more than 5 carbons, because no activity was observed with dulcitol (galactitol), which has the hydroxyl group of C-2 in the L- and the hydroxyl group of the C-3 in the D-configuration, but is a six-carbon sugar alcohol. Also, no activity was observed with the C-6 sugars D-mannitol and L-gulitol (D-sorbitol) where the hydroxyl group of C-2 is in the L-configuration. The L-xylulose reductase of H. jecorina was less spe-
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cific; it only required a hydroxyl group at C-2 in the l-configuration in the sugar alcohol, and was active with C-6 sugar alcohols. In the reverse direction, activity with the 2-ketose sugar was preferred when the hydroxyl group of C-3 was in the l-configuration. We saw highest activity with l-xylulose and d-ribulose but only low activity with d-xylulose. Also in this direction the enzyme seemed to be specific for sugars with not more than 5 carbons, because no activity was observed with d-sorbose and d-psicose, both 2-keto sugars with the hydroxyl group in C-3 in d-configuration but with 6 carbons.

This is another difference to the l-xylulose reductase from mold H. jecorina. The mold enzyme also converted sugar alcohols where the C-2 is in l-configuration but the hydroxyl of C-3 can be either l or d (4), i.e. the H. jecorina enzyme is less specific also in this direction.

The kinetic properties of the l-xylulose reductase from A. monospora were more similar to the properties of the d-arabinitol dehydrogenases of the yeasts P. stipitis (23) and C. tropicalis (22) than to the l-xylulose reductase of H. jecorina (4). The yeast enzymes are NADH-specific and have activity with d-arabinitol. The A. monospora enzyme, however, is different from the d-arabinitol dehydrogenases because it has high activity with xylitol. The activities of the d-arabinitol dehydrogenases with xylitol were less than 10% of the activities with d-arabinitol.

According to Hallborn et al. (23) the P. stipitis d-arabinitol dehydrogenase can participate in d-xylulose metabolism, i.e. catalyze the reaction from xylitol to d-xylulose. However, no evidence was given that d-xylulose rather than l-xylulose was produced from xylitol. Another possible interpretation of the finding that the d-arabinitol dehydrogenases from C. tropicalis and P. stipitis have activity with xylitol is that these enzymes catalyze the same reaction as the A. monospora enzyme, i.e. have a preference for sugar alcohols with the hydroxyl of the C-2 in l- and the C-3 in the d-configuration and convert xylitol to l-xylulose. This would mean that the yeast l-xylulose reductase and the d-arabinitol dehydrogenase are very similar enzymes with partially overlapping activities. This is different for the enzyme from mold H. jecorina where the l-xylulose reductase is very similar to the d-mannitol dehydrogenase (4).

By Northern analysis we could show that ALX1 is most strongly expressed in the yeast A. monospora when grown on l-arabinose. This indicates that it is part of the natural l-arabinose pathway of this yeast. The gene was expressed on all carbon sources studied, which suggests that it might also have some other role in addition to l-arabinose catabolism.

The two pentose sugars that are most abundant in plants are d-xylulose and l-arabinose. The pathways for their catabolism are therefore relevant for micro-organisms that are living on decaying plant material. They are also relevant in the applied conversion of plant biomass, a cheap raw material, to bulk products, such as fuel ethanol. For the catabolism of l-arabinose, two microbial pathways are known, a bacterial and a fungal pathway. In both pathways l-arabinose is converted to d-xylulose 5-phosphate; however, the pathways are distinctly different (Fig. 1).

Both pathways were functionally expressed in S. cerevisiae, and the resulting strains were able to convert l-arabinose to ethanol under oxygen-limited or anaerobic conditions. With the bacterial pathway an ethanol production rate of up to 0.08 g of ethanol/g dry mass h\(^{-1}\) was obtained (24), whereas with the functionally expressed fungal pathway the ethanol production rate was more than an order of magnitude lower (5). The low fermentation rate with the fungal pathway might be explained by an imbalance of redox cofactors. The fungal l-arabinose pathway goes through two reductions that are NADPH-linked and two oxidations that are NAD\(^{+}\)-linked. S. cerevisiae might not have the capability to cope with the resulting imbalance of redox cofactors. With the expression of an NADH-l-xylulose reductase instead of an NADPH-dependent enzyme, as described in this work, this cofactor imbalance is at least partly avoided.

The microbial catabolism of the pentose sugar d-xylulose has similarities to the catabolism of l-arabinose. d-Xylulose is similar to l-arabinose, catalyzed by different pathways in bacteria and fungi. The bacterial pathway, consisting of xylitol isomerase and xylulokinase, is redox neutral, whereas the fungal pathway goes through a reduction (xylulose reductase) and an oxidation (xylitol dehydrogenase). In fungi the reductions in both pathways are NADPH-linked and the oxidations NAD\(^{+}\)-linked. An exception is the anaerobic fungus Piromyces sp. E2, which contains a xylitol isomerase as in the bacterial pathway (25).

When the reductions use NADPH and the oxidations NAD\(^{+}\), a cofactor imbalance is generated, which hinders the catabolism especially under anaerobic conditions. To facilitate d-xylulose catabolism yeasts have developed various approaches. P. stipitis has an unspecified aldose reductase that also accepts NADH as a cofactor (26), and Kluveromyces lactis has an NADP\(^{+}\)-utilizing glyceraldehyde-3-phosphate dehydrogenase that can balance the redox cofactors (20). The cofactor imbalance in the l-arabinose pathway is at least partly avoided in the yeast A. monospora by having an NADH-l-xylulose reductase.

We now have indications that the fungal pathway for the catabolism of l-arabinose can be divided into two subclasses, one for yeast and one for mold, because of the different cofactor requirements. The l-xylulose reductases described for the molds P. chrysogenum (1), A. niger (27), and H. jecorina (4) are NADPH-specific. The only l-xylulose reductase from yeast, as described in this work, is strictly NADH-specific. It remains an open question whether other yeast species use an enzyme with a similar cofactor requirement.

Acknowledgment—We thank Helena Simolin for the HPLC analysis.

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J. Biol. Chem. 2004, 279:14746-14751.
doi: 10.1074/jbc.M312533200 originally published online January 21, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M312533200

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