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Expression of the *Escherichia coli* Xylose Isomerase Gene in *Saccharomyces cerevisiae*

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Transformation of *Saccharomyces cerevisiae* by yeast expression plasmids bearing the *Escherichia coli* xylose isomerase gene leads to production of the protein. Western blotting (immunoblotting) experiments show that immunoreactive protein chains which comigrate with the *E. coli* enzyme are made in the transformant strains and that the amount produced parallels the copy number of the plasmid. When comparable amounts of immunologically cross-reactive xylose isomerase protein made in *E. coli* or *S. cerevisiae* were assayed for enzymatic activity, however, the yeast protein was at least 10^3-fold less active.

Cellulose, lignin, and hemicellulose are the three major biopolymers in wood and agricultural crop residues (21). Of these, hemicellulose has the least value as intact material and is most readily converted, by simple acidic hydrolysis, into its monomeric building blocks. Chief among these is D-xylose, an aldopentose. In principle, bioconversion of xylose to ethanol should be possible by anaerobic fermentation; however, naturally occurring yeasts capable of ethanol fermentation do not utilize xylose, and organisms which utilize xylose do so under aerobic conditions. Several solutions to this problem have been proposed. These include wider searches in nature for xylose-fermenting organisms; mutagenesis of xylose utilizers such as *Pachysolen tannophilus* (24), *Candida tropicalis* (13), and similar yeasts; and finally, genetic engineering of well-characterized strongly fermenting yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* to introduce a xylose utilization pathway.

The inability of yeasts to ferment xylose may result from the lack of a single enzymatic step, since the related ketopentose xylulose is fermented to ethanol by some strains of *S. cerevisiae* (9, 26). In *Escherichia coli*, the interconversion of xylose and xylulose is catalyzed by the enzyme xylose isomerase (11, 16), an enzyme whose structural gene has been cloned and sequenced (6, 15, 23). Using yeast expression plasmid vectors, we have introduced the *E. coli* xylose isomerase gene into *S. cerevisiae*. We report here our initial results on characterization of the xylose isomerase protein made in *S. cerevisiae*.

MATERIALS AND METHODS

Strains. *E. coli* RR1 (3) was used for propagation of plasmids. Bacterial strains and bacteriophages used for DNA sequence analysis of plasmid pXI-1 are described by Sanger et al. (22). *S. cerevisiae* EG1-103 (*MATa ura3 trp1 leu2*) and Kn-79 (*MATα leu2 trp1*) were obtained from Vivian MacKay. Yeast transformation was carried out by the method of Beggs (2).

Enzymes and chemicals. Restriction enzymes, exonuclease III, S1 nuclease, the Klenow fragment of DNA polymerase, T4 DNA ligase, and bacterial alkaline phosphatase were obtained from commercial suppliers and used as specified by the manufacturer. Xylose and xylulose were obtained from Sigma Chemical Co. Radiochemicals were from Amersham Corp.

Plasmids. The *E. coli* xylose isomerase structural gene is contained in plasmid pXI, which has been described previously (23). Construction of plasmid pXI-1 (see Fig. 1) involved a brief treatment with exonuclease III followed by S1 nuclease to generate deletions at the unique *Sst*II site on pXI by the method of Henikoff et al. (12). Phosphorylated *Eco*RI linkers in a fivefold molar excess were ligated to the deleted molecules, and the DNA was recircularized with T4 DNA ligase and cloned into *E. coli* RR1. Plasmids containing deletions between 20 and 50 base pairs in size were identified by electrophoresis on a 1.5% agarose gel, and the sequence of pXI-1 was obtained by the dideoxy sequencing method of Sanger et al. (22).

Yeast expression plasmids were prepared by fusing the *E. coli* xylose isomerase gene to the *ADH1* promoter and terminator regions in the yeast expression vector pAAH5 (1). Plasmid pXI-1 was digested with *Eco*RI and *Bgl*II to yield a fragment which contains the xylose isomerase gene. This fragment was treated with Klenow polymerase to generate flush ends; it was then treated with phosphorylated *Hin*dIII linkers and ligated into a *Hin*dIII-cut pAAH5 vector which had been dephosphorylated with bacterial alkaline phosphatase. Plasmid pScXI-AAH5 contains the xylose isomerase gene fused in the correct orientation to the *ADH1* gene promoter and terminator regions. We prepared other yeast expression plasmids from pScXI-AAH5 by cloning either a *Bam*HI or a *Sph*I fragment containing the *ADH1* promoter and terminator flanking xylose isomerase gene into high-copy-number plasmid pC1/1 or centromere plasmid pTC3. The resultant plasmids are shown in Fig. 3. The vector pC1/1 was obtained from Elton T. Young, and pTC3 was obtained from Dan Allison.

Determination of xylose isomerase RNA. A mid-log-phase yeast culture grown in 100 ml of yeast nitrogen base liquid medium under leucine selection was harvested and washed once with cold glass-distilled water. After centrifugation, the pellet was suspended in 1/30 the original volume of extrac-

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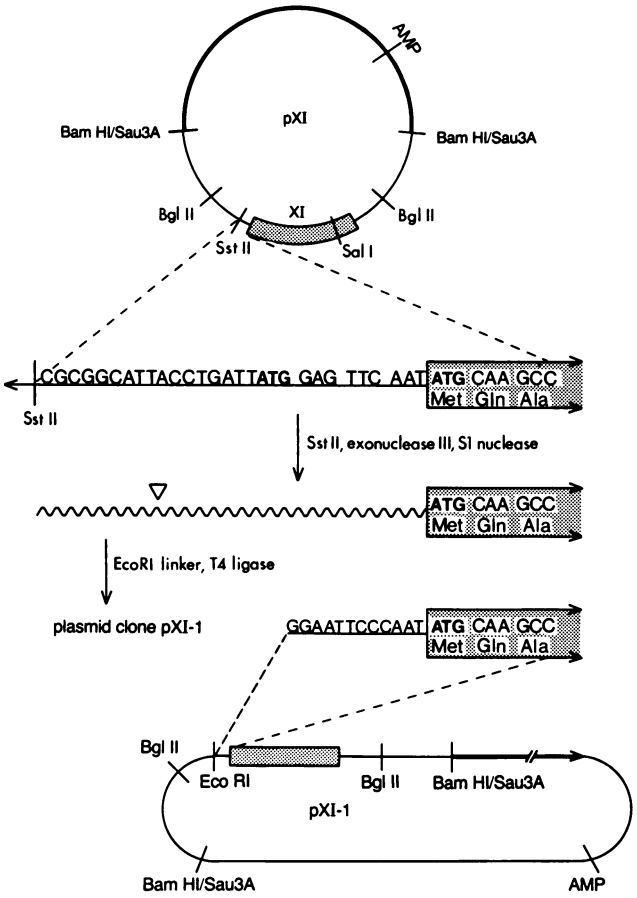

FIG. 1. Construction of plasmid pXI-1. Deletions were generated at the unique *Sst*II site of plasmid pXI with exonuclease III and S1 nuclease. After *Eco*RI linker addition and recircularization with T4 DNA ligase, deleted molecules were cloned into *E. coli*. The deletion in plasmid clone pXI-1 was shown by dideoxy sequencing to extend to within 4 base pairs of the initiator codon.

tion buffer containing 0.15 M NaCl, 0.05 M Tris hydrochloride (pH 7.4), 0.005 M EDTA, and 5% sodium dodecyl sulfate (SDS). An equal volume of phenol-chloroform-isoamyl alcohol (50:50:1, vol/vol/vol) was immediately added. The mixture was vortexed vigorously in the presence of glass beads for 2 min and then centrifuged at 4,000 × *g* for 5 min. The aqueous phase was reextracted with an equal volume of phenol-chloroform-isoamyl alcohol (50:50:1) and then extracted with chloroform. RNA present in the aqueous phase was precipitated with 2 volumes of cold 95% ethanol containing 0.2 M sodium acetate. Northern blot (RNA blot) analysis was done by the method of Thomas (25). A DNA fragment containing only the xylose isomerase gene was used to hybridize the yeast RNA blotted onto the nitrocellulose paper. The probe was radioactively labeled with [α-^{32}P]dATP by the nick translation method described by Maniatis et al. (17).

Preparation of antiserum. Antiserum against the *E. coli* xylose isomerase was prepared as follows. Xylose isomerase from the chromatofocusing step (23) was further purified by preparative SDS-polyacrylamide gel electrophoresis (14). The gel was stained with 0.2% Coomassie blue in 50% methanol. The major band (xylose isomerase) was cut from the gel, macerated, and emulsified in 5 ml of 50% complete Freund adjuvant in normal saline. The preparation was injected subcutaneously into a New Zealand White male rabbit. At 3 weeks later, a booster identical to the preparation in the first injection, except for replacement of the Freund complete adjuvant with incomplete adjuvant, was prepared. Antiserum was harvested 5 weeks after the first injection was administered.

Western blot (immunoblot) analysis. Yeast cells containing the plasmids were grown overnight in yeast nitrogen base liquid medium under leucine or tryptophan selection at 30°C to an optical density at 600 nm of approximately 2.0. After being centrifuged at 1,000 × *g* for 10 min, the cell pellet was washed with glass-distilled water and suspended in 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9)–0.01 M $MgCl_2$–0.005 M dithiothreitol–0.001 M phenylmethylsulfonyl fluoride. Cells were broken open by being vigorously vortexed with an equal volume of glass beads in 1-min bursts while being kept cold. Extracts for Western blot analysis were boiled in 1% SDS–5% β-mercaptoethanol–63 mM Tris (pH 6.8)–10% glycerol, and the proteins were separated on a SDS–10% polyacrylamide gel. The proteins were then electrophoretically transferred to nitrocellulose paper, and the paper was incubated with rabbit antiserum against the purified *E. coli* xylose isomerase and then treated with affinity-purified ^{125}I-labeled protein A. Autoradiography was at −70°C. Protein determinations were done by the Bradford assay (5).

Xylose isomerase enzymatic activity assay. Xylose isomerase activity was determined by measuring the extent of [^{14}C]xylose conversion to [^{14}C]xylulose. The assay mixture (10 μl) contained 0.001 M xylose, 0.2 μCi of [^{14}C]xylose (specific activity, 76 mCi/mmol; Amersham), 0.01 M HEPES (pH 7.9), 0.01 M $MgCl_2$, 0.005 M dithiothreitol, 0.001 M phenylmethylsulfonyl fluoride, and 25 μg of *E. coli* RR1 extract protein. The reaction was initiated by addition of the extract to be measured. Reaction mixtures were incubated for 2 h at 37°C, chilled on ice, and spun for 5 min at 15,000 rpm in a Microfuge (Beckman Instruments). A 5-μl portion of the supernatant was then spotted on a polyethyleneimine thin-layer chromatography plate (Brinkman CEL 30 PEI/UV). The solvent system used for the separation of [^{14}C]xylose from [^{14}C]xylulose was butanol-acetone-water (4:5:1, vol/vol/vol). The [^{14}C]xylulose spots were cut from the sheets, and the radioactivity was counted.

RESULTS

Construction of plasmids for expression of *E. coli* xylose isomerase gene in *S. cerevisiae*. The xylose isomerase gene is contained within a 1.6-kilobase *Bgl*II fragment in plasmid pXI (Fig. 1). For direct expression of the *E. coli* xylose isomerase gene in *S. cerevisiae*, we placed the coding sequence between the *ADH1* promoter and terminator regions in yeast expression vector pAAH5 (Fig. 2) (1).

Plasmid pXI was linearized at a unique *Sst*II restriction site which is 30 base pairs upstream of the initiator ATG codon of the xylose isomerase gene. The linear plasmid was briefly treated with exonuclease III and subsequently with S1 nuclease to remove the sequences between the *Sst*II site and the xylose isomerase gene. The deleted DNA molecules were recircularized after *Eco*RI linker addition and cloned into *E. coli* (Fig. 1). The deletion in pXI-1 was found by dideoxy sequencing to extend to within 4 base pairs of the initiator codon. Subsequently the *Eco*RI-*Bgl*II fragment of pXI-1 was converted to a *Hin*dIII fragment by fill-in DNA synthesis and *Hin*dIII linker attachment. This was inserted into the *Hin*dIII cloning site of the yeast expression vector

pAAH5 (Fig. 2), and the recombinant plasmid was cloned in *E. coli*. Restriction analysis of quick plasmid DNA preparations identified bacterial isolates containing the proper orientation of the xylose isomerase gene for transcription from the yeast *ADH1* promoter.

Yeast expression plasmids such as pAAH5, which contain the 2μm circle origin of replication and a nearby fragment designated *REP3*, are maintained at approximately 30 copies per cell in most yeast strains (7). Other expression plasmids containing the xylose isomerase gene, which would be present in much higher (ca. 200) or lower (1 or 2) copy number were constructed (Fig. 3). Yeast plasmid vector pC1/1 has two features contributing to an elevated copy number in *S. cerevisiae*. Like its parent, pJDB219, it contains the entire 2μm sequence and a truncated *LEU2* gene as selectable marker (2). A *Bam*HI fragment containing the yeast *ADH1* promoter and terminator as well as the xylose isomerase gene was removed from pScXI-AAH5 and introduced into the unique *Bam*HI site of pC1/1 (Fig. 3a).

A plasmid which contains a yeast centromere sequence limits replication and stabilizes the copy number at a low number (10). The plasmid pTC3 carries the *CEN3* sequence, the *ARS1* replication origin, and *TRP1* as a selectable marker. To obtain a low-copy-number xylose isomerase expression plasmid, an *Sph*I fragment containing the XI gene and flanking *ADH1* regulatory sequences was removed from pScXI-AAH5 and inserted at the unique *Sph*I site in pTC3 (Fig. 3b).

Isolation and characterization of *S. cerevisiae* transformants containing the xylose isomerase gene. Several *S. cerevisiae* strains were transformed with xylose isomerase-containing plasmids and with parent vectors by selecting for leucine or tryptophan prototrophs. The transformant clones were then tested for their ability to grow on xylose as the sole carbon source. Transformants containing the xylose isomerase gene in low, medium, or high copy number were all unable to grow on yeast nitrogen base medium containing 1 to 10% xylose regardless of the yeast strain used.

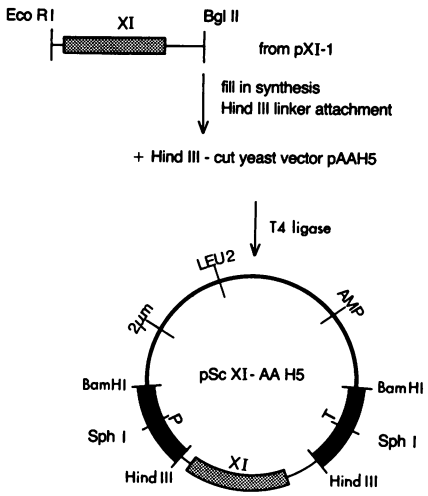

FIG. 2. Construction for *E. coli* xylose isomerase gene expression in *S. cerevisiae*. An *Eco*RI-*Bgl*II fragment from pXI-1 containing the xylose isomerase gene was converted to a *Hin*dIII fragment by fill-in synthesis with Klenow polymerase and *Hin*dIII linker attachment. This fragment was then fused to the *ADH1* promoter (P) and terminator (T) regions at a unique *Hin*dIII site in the yeast expression vector pAAH5. pScXI-AAH5 should be present in the yeast cell at approximately 30 copies per cell.

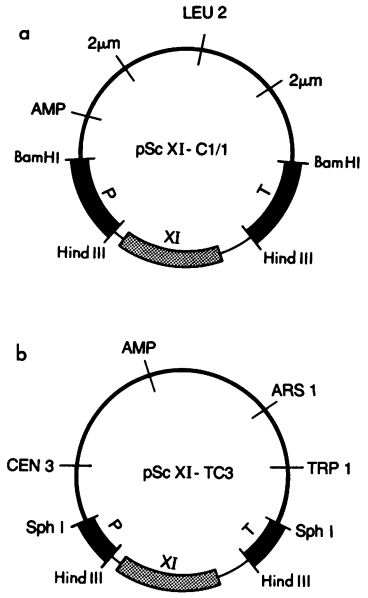

FIG. 3. Structure of high- or low-copy-number yeast expression plasmids containing the *E. coli* xylose isomerase gene flanked by the *ADH1* promoter and terminator regions. Approximately 200 copies of pScXI-C1/1 (a) are present per yeast cell, whereas pScXI-TC3-transformed yeast cells (b) have 1 or 2 copies of the plasmid.

Northern blot analysis (25) was performed on a pScXI-AAH5 transformant of *S. cerevisiae* EG1-103 to determine whether cells transformed with the recombinant plasmid transcribe mRNA from the *ADH1* promoter. Total xylose isomerase RNA, made in transformants containing the plasmid pScXI-AAH5 grown under leucine selection, was detected by hybridization of total RNA from this transformant with the cloned xylose isomerase gene (data not shown). The size of this RNA (ca. 1.5 kilobases) was that expected for a transcript initiated from the *ADH1* promoter and terminated within the *ADH1* terminator sequence. These results indicate that the inability of the yeast transformants to grow on xylose is not due to a failure of the cells to transcribe the xylose isomerase gene from the *ADH1* promoter.

The level of in vivo translation of the xylose isomerase mRNA made in *S. cerevisiae* transformants containing the xylose isomerase gene was measured by Western blot analysis (8). Extracts of mid-log-phase yeast cultures grown under leucine or tryptophan selection were prepared by breaking the cells with glass beads. The preparations were solubilized in 1% SDS, and the proteins were electrophoresed on an SDS-polyacrylamide gel (14) and electrophoretically transferred to nitrocellulose. Probing with anti-xylose isomerase antibody showed the presence of an immunologically cross-reactive xylose isomerase polypeptide in extracts of *S. cerevisiae* Kn-79 containing pScXI-AAH5 and pScXI-C1/1 (Fig. 4, lanes 3 and 5). The antibody cross-reacting material comigrated with xylose isomerase from an *E. coli* lysate which contains the plasmid pXI (lane 1). Yeast transformants containing the higher-copy-number pC1/1-derived plasmids always made substantially more cross-reacting material than did extracts from cells containing pAAH5-derived plasmids, regardless of the yeast strain used. A longer exposure of the autoradiogram from this experiment demonstrated that a small amount of the polypeptide was produced in yeast cells transformed with the

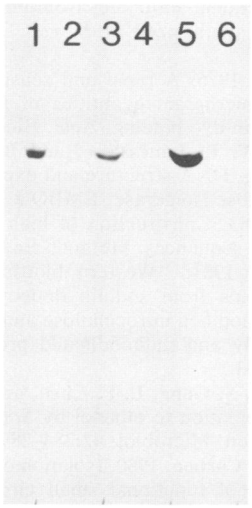

FIG. 4. Western blot analysis of total cell proteins of *S. cerevisiae*. Lane 1: 25 μg of protein extracted from *E. coli* cells containing plasmid pXI mixed with 250 μg of protein extracted from *S. cerevisiae* cells containing plasmid pAAH5. Lanes 2 to 5: 250 μg of yeast protein extracted from *S. cerevisiae* cells grown under leucine or tryptophan selection containing plasmid pScXI-TC3 (lane 2), pScXI-AAH5 (lane 3), pAAH5 (lane 4), pScXI-C1/1 (lane 5), or pC1/1 (lane 6).

single-copy plasmid pScXI-TC3 (data not shown). Thus the level of xylose isomerase protein expression parallels the copy number of the recombinant plasmid. Yeast transformants containing pScXI-C1/1 produce approximately 20% as much cross-reacting xylose isomerase protein as do *E. coli* transformants containing pXI when expressed per milligram of extract protein.

The experiments described above are for yeast whole-cell extracts not subjected to centrifugation until after solubilization in 1% SDS. When yeast extracts were centrifuged at 10,000 × *g* for 5 min before being boiled in SDS, however, approximately half of the xylose isomerase protein was removed from the extract, indicating that a major portion of the protein is present in a particulate form. In contrast, the xylose isomerase produced in *E. coli* is a soluble protein.

A xylose isomerase assay (described in Materials and Methods) was developed to measure the enzymatic activity of the protein made in yeast transformant clones. Maximal conversion of [^{14}C]xylose to [^{14}C]xylulose under conditions of the assay (13%) was obtained by using approximately 100 ng of purified *E. coli* xylose isomerase. Extracts were prepared from transformant yeast clones by breaking cells with glass beads in the presence of reducing agent and protease inhibitors. Low levels of enzymatic activity were detected in yeast extracts containing the xylose isomerase gene in the vectors pAAH5 and pC1/1, but in no case did a yeast extract approach the level of enzymatic activity observed for bacterial transformants containing pXI.

Although a substantial amount of the immunologically cross-reactive xylose isomerase protein was removed from whole-cell extracts by centrifugation at 10,000 × *g*, the enzymatic activity was not decreased by this treatment, indicating that like the authentic *E. coli* enzyme, the active xylose isomerase molecules made in *S. cerevisiae* are soluble. However, these data suggest that a major portion of the heterologously made xylose isomerase protein is inactive.

Since the level of enzymatic activity of yeast crude extracts is low, determination of the specific activity is difficult. One possibility is to compare the levels of enzyme activity obtained in yeast crude extracts with that seen in bacterial lysates and then to correlate these results with the amounts of xylose isomerase protein observed by Western analysis. The xylose isomerase activity of an *E. coli* crude extract was measured (Fig. 5). This bacterial extract was also assayed in the presence of an extract of *S. cerevisiae* Kn-79 transformed by vector pC1/1 to test for possible inhibitor substances in yeast extracts. The decrease in enzyme activity in an *S. cerevisiae* environment compared with an *E. coli* environment was less than twofold. The xylose isomerase activity of a yeast pScXI-C1/1 transformant in *S. cerevisiae* Kn-79 is also shown in Fig. 5.

The ratio of enzyme activity to total protein is at least 5 × 10^3-fold greater for *E. coli* than for a yeast extract, and yet these lysates had an equivalent Western blot signal when only five times as much yeast protein was added (Fig. 4, lanes 1 and 5). These crude estimates suggest that the yeast protein is at least 10^3-fold less active or that only a small percentage of molecules made in *S. cerevisiae* have any activity.

DISCUSSION

We have expressed the *E. coli* xylose isomerase gene in *S. cerevisiae* by using sequences from the yeast *ADH1* gene as promoter and terminator. Although the level of immunologically cross-reactive protein produced in *S. cerevisiae* varied with the presumed copy number of the vector, the enzymatic activity of the protein was low in all cases. Although we

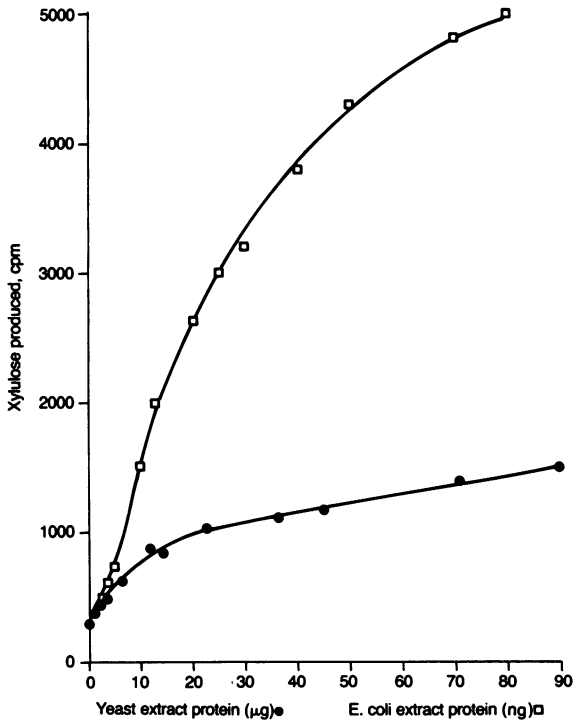

FIG. 5. Xylose isomerase enzymatic activity as a function of added bacterial or yeast extract protein. Various amounts of the extracts were added to incubation mixtures containing the substrate [^{14}C]xylose. The product of the reaction ([^{14}C]xylulose) was separated from the substrate by polyethyleneimine thin-layer chromatography in butanol-acetone-water, and the radioactivity in the [^{14}C]xylulose spots was counted.

cannot accurately measure the specific activity of the xylose isomerase produced in *S. cerevisiae* without further purification, we can normalize the observed enzymatic activities of *S. cerevisiae* and *E. coli* lysates for the amount of cross-reactive polypeptide measured by Western blot analysis. When this is done, the protein produced in *S. cerevisiae* appears to be at least a 10^3-fold less active. Although we cannot distinguish between lowered enzymatic activity for all molecules and a mixture of inactive and active molecules, we favor the latter explanation, because centrifugation removes a substantial portion of the antibody cross-reacting material but the enzymatic activity remains soluble.

There are a number of reasons that could explain the inactivity of the enzyme made in *S. cerevisiae*. These include improper folding of the protein, the internal pH of the yeast cells, posttranslational modifications of the protein, and inter- or intramolecular disulfide bridge formation. In attempts to increase enzyme activity in yeast cells, extracts have been prepared in a variety of ways. When the pH of the extraction buffer was raised from 7.4 to 8.5, there was an increase in activity; this increase, however, was only two- to threefold (20). We have examined the possibility of posttranslational modifications of the *S. cerevisiae* or *E. coli* enzyme by comparison of the mobility of the two proteins on isoelectric focusing gel electrophoresis under denaturing conditions. No substantial charge differences were observed (data not shown). The presence of inter- or intramolecular disulfide bridge formation in a protein can be determined by a shift in mobility of the protein when subjected to SDS-polyacrylamide gel electrophoresis under reducing and nonreducing conditions. However, Western blot analysis of bacterial or yeast extracts prepared under nonreducing or reducing conditions did not reveal any differences in the pattern of mobility of the immunologically cross-reacting proteins (data not shown).

Similar examples of inactive and aggregated proteins produced in heterologous systems have also been reported (4, 18, 19). Some of these proteins have regained activity after modifications of the proteins by in vitro extraction procedures, e.g., inactive prochymosin made in *E. coli* reassembled in vitro into active enzyme after being treated with urea and alkali (19). Perhaps directed renaturation of the yeast enzyme in vitro could result in an active enzyme, suggesting that the protein may be improperly folded in vivo. Finally, we have not ruled out the possibility that the absence of an essential cofactor or metal ion in *S. cerevisiae* is essential to the activity of xylose isomerase made in vivo in yeast cells.

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