Alcohol dehydrogenase II and fructose-1,6-bisphosphatase appear to be co-regulated in wild-type yeast

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An activity gel assay for fructose-1,6-bisphosphatase (FBP), the enzyme catalyzing the final step in gluconeogenesis in yeast, has been developed which can be used in conjunction with spectrophotometric assays to show that it is tightly co-regulated with the inducible alcohol dehydrogenase, ADHII. Both enzymes are repressed coordinately in aerobically grown yeast by the addition of high levels of glucose or ethanol, and induced on minimal medium by the addition of yeast extract. A mutant deficient in FBP segregates independently of the ADHII structural gene locus. This phenomenon is of interest because of the discovery of Ciriacy [(1979) Mol. Gen. Genet. 176, 427–431] of mutants (*ccr*, or carbon catabolite repression) which repress both FBP and ADHII simultaneously, along with several other enzymes.

Activity gel electrophoresis *Gluconeogenesis* *Regulation*

1. INTRODUCTION

When grown under anaerobic or low-oxygen conditions, the yeast *Saccharomyces cerevisiae* quickly exhausts metabolizable sugars in a dilute medium and produces ethanol by fermentation. If the medium is then aerated, the ethanol is rapidly utilized. A number of enzyme systems, repressed under anaerobic conditions, are induced by aeration, and this induction can be prevented by higher levels of sugars. Many mechanisms have been suggested for this induction and repression (review [1]). Regardless of the mechanisms of induction or repression, the enzymes which are induced tend to be those which enable ethanol to be rapidly and efficiently utilized.

These enzymes include the inducible alcohol dehydrogenase, ADHII, the kinetics of which facilitate the conversion of ethanol to acetaldehyde [2]. Enzymes of the glyoxylate cycle then convert acetaldehyde to succinate, which can enter the Krebs cycle. Some of these enzymes have been shown to be induced by aeration. Finally, gluconeogenesis, essential for growth on ethanol as a carbon source, involves 3 cytoplasmic enzymes that are not involved in glycolysis: malate dehydrogenase, phospho*enol*pyruvate carboxykinase and fructose-1,6-bisphosphatase.

Biochemical studies have shown that all the enzymes of the glyoxylate cycle appear to be present in the cytoplasm of yeast cells [3]. We have recently investigated a mutant deficient in pyruvate carboxylase which adds some genetic evidence to these observations and further suggests that the glyoxylate and Krebs cycles may be coupled in ethanol-grown yeast through the exchange of succinate and malate across the inner mitochondrial membrane [4].

We show here that two important enzymes involved in the pathway of ethanol utilization are in fact tightly and coordinately regulated in the wild type cell. These enzymes are the inducible alcohol dehydrogenase (ADHII) (EC 1.1.1.1) and fructose-1,6-bisphosphatase (FBP) (EC 3.1.3.11). The structural genes for these enzymes appear not to be linked, although this cannot yet be stated with certainty.

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2. MATERIALS AND METHODS

2.1. *Yeast strains*

Two wild type strains were employed in the determination of enzyme activities. Strains X2180-1A and X2180-1B (*MATa* and *MATα*, respectively, otherwise *SUC2 mal gal2 CUP1*) were obtained from the Yeast Genetics Stock Center, Berkeley. An alcohol dehydrogenase negative strain derived from these strains, XW942-1B, lacks the two cytoplasmic and the mitochondrial alcohol dehydrogenase (*MATα adr adc adm*). Strains XW68-1A and XW68-1C (*MATα* and *MATa*, respectively) are deficient in fructose-1,6-bisphosphatase (*fbp*).

2.2. *Media employed*

Media used were complete [2% Bacto Peptone (Difco, w/v), 1% yeast extract (Difco, w/v)] and minimal [0.75% Yeast Nitrogen Base without amino acids (Difco, w/v)]. To these basic media were added dextrose or ethanol as carbon sources in various concentrations. Yeast extract was added to minimal medium in various concentrations in order to quantify the difference between complete and minimal medium. To convert cells to a petite (aerobic respiration deficient) phenocopy, 1 ml/l of a 1 mg/ml solution of antimycin A (Sigma) in 95% ethanol (filter sterilized) was added after autoclaving [5].

2.3. *Growth conditions*

All cells were grown at 30°C, either on plates or in 250 ml flasks containing 100 ml of liquid medium in New Brunswick air bath incubator shakers set at 250 rpm.

2.4. *Mutagenesis*

Mutations were induced in log-phase cells at a concentration of 10^7 cells/ml in complete medium with 2% (w/v) dextrose as carbon source. Cells were treated with 3% (v/v) ethyl methane sulfonate (Sigma) for 1 h, washed in 50 mM K-PO_4 buffer (pH 7.0), then resuspended for 15 min in 6% sodium thiosulfate. After two more washes in buffer, they were plated to give 100–200 viable cells per plate [6].

2.5. *Mutant isolation*

Strain XW942-1B, the alcohol dehydrogenase negative strain, was mutagenized with ethyl methane sulfonate, and plated on minimal medium with dextrose as a carbon source. This had the effect of eliminating petites, which cannot survive in an alcohol dehydrogenase negative strain [6], and also amino acid auxotrophs, which cannot survive on minimal medium. These colonies were then replicated to minimal medium with pyruvate (1%, w/v, sodium pyruvate) as a carbon source. Mutants unable to grow on this latter medium were crossed to wild type for two generations, in order both to remove the mutant alcohol dehydrogenase alleles and to produce strains which segregated regularly and sporulated readily. A number of these mutants are still under investigation; one, which was unable to grow on any non-fermentable substrate although it was not a petite, was discovered to be deficient in FBP. Segregants of this strain, from cross XW68, were investigated further.

2.6. *Genetic analysis*

Standard procedures [7] were used for mating, sporulation, tetrad dissection and tetrad analysis.

2.7. *Visualization of enzyme patterns*

Enzymes were separated by horizontal starch gel electrophoresis, and alcohol dehydrogenase patterns were visualized by standard techniques [6]. A coupled assay was devised to visualize the FBP. The activity stain consisted of 50 mM imidazole-HCl buffer (pH 7.0), containing 10 mM $MgCl_2$, 100 mM KCl, 1 mM ethylene diamine tetraacetic acid (EDTA), disodium salt, 0.1 mM fructose bisphosphate and 0.25 mM NADP. To each 100 ml aliquot of this stain was added 0.5 units of phosphoglucose isomerase and 0.5 units of glucose-6-phosphate dehydrogenase, 25 mg nitro blue tetrazolium and 4 mg phenazine methosulfate (all from Sigma). The gel was developed in the dark overnight at room temperature, rinsed several times in glass-distilled water, and preserved and rendered transparent by immersion in glycerol for several hours.

2.8. *Enzyme assays*

Isocitrate lyase, malate dehydrogenase and phospho*enol*pyruvate carboxykinase were assayed spectrophotometrically according to published procedures [8–10]. Malate dehydrogenase was

assayed by NADH formation, the direction it would be expected to proceed during gluconeogenesis, and shows activity levels approximately comparable to those of the other enzymes. The spectrophotometric assay of FBP posed a special problem, because this enzyme is markedly inhibited by substrate levels in excess of 0.1 mM and there are high levels of fructose-1,6-bisphosphate present in crude cellular extracts (in preparation). While the substrate could be removed by dialysis, this also had the effect of reducing the enzyme activity by an unknown but variable amount. The spectrophotometric assay mix was the same as the activity gel stain but without nitro blue tetrazolium and phenazine methosulfate. A method for measuring activity levels in the crude extract without dialysis was devised as follows: concentrated (70%) perchloric acid was added to small aliquots of crude extract to a final concentration of 0.6 N. The extract was kept on ice for 20 min, then centrifuged for 5 min in a Fisher microcentrifuge. The supernatant was carefully removed by pipetting, and adjusted to pH 7.0 with 1 M KOH. Fructose bisphosphate levels in this deproteinated extract were measured spectrophotometrically. Levels in the crude extract varied markedly depending on growth conditions, ranging to as high as 12 mM. Using this information, levels of fructose bisphosphate were adjusted in the reaction mix to give a final level of 0.1 mM when the crude extract was added. Levels of fructose 6-phosphate and glucose 6-phosphate, the intermediates in the coupled assay, were not detectable in the deproteinated extract. Protein was determined by the method of Lowry et al. [11].

3. RESULTS

3.1. *ADHII and FBP are co-regulated over a range of growth conditions*

Fig.1 shows the induction of ADHII and FBP in cells grown to plateau on liquid minimal medium with the addition of varying amounts of yeast extract. It will be seen that appreciable amounts of both enzymes are synthesized at concentrations of yeast extract of 0.5% and above. It has been shown elsewhere [12] that the induction of ADHII is not correlated directly with the disappearance of glucose from the medium. Since it is induced under the same conditions as FBP, it would appear that

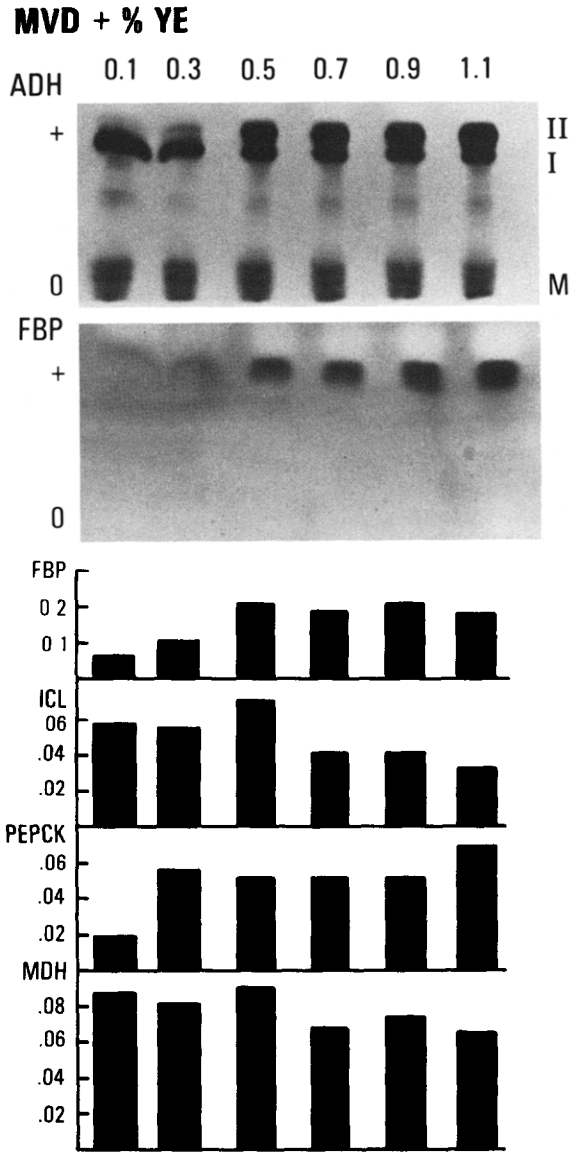

Fig.1. Effect on enzyme levels of increasing yeast extract concentration in otherwise minimal medium with dextrose as carbon source. The top gel shows the alcohol dehydrogenase patterns. II, I and M refer to the two cytoplasmic isozymes ADHII and ADHI and to the mitochondrial isozyme ADHM. The second gel shows a slice from the same gel stained for FBP activity. The histograms below show FBP, isocitrate lyase (ICL), phospho*enol*pyruvate carboxykinase (PEPCK) and malate dehydrogenase (MDH) activities, expressed as μmol NADP O_2 NAD^+ reduced or NADH oxidized/min per mg protein (see section 2).

the induction of the latter enzyme is also not directly correlated with glucose levels.

Fig.2 shows the effect of altering glucose levels in complete medium. At high glucose levels, induction of the two enzymes is coordinately, though not markedly, reduced. The apparent marked reduction of FBP levels seen on the gel at 8% glucose is not seen in the spectrophotometric assay.

Fig.3 shows the effect of altering ethanol levels in complete medium. Growth conditions are otherwise the same. ADHII and FBP levels are markedly reduced at levels of ethanol greater than 5%. It will be seen from the figure that levels of ADHI, the less anodally migrating of the two major alcohol dehydrogenase isozymes, are not

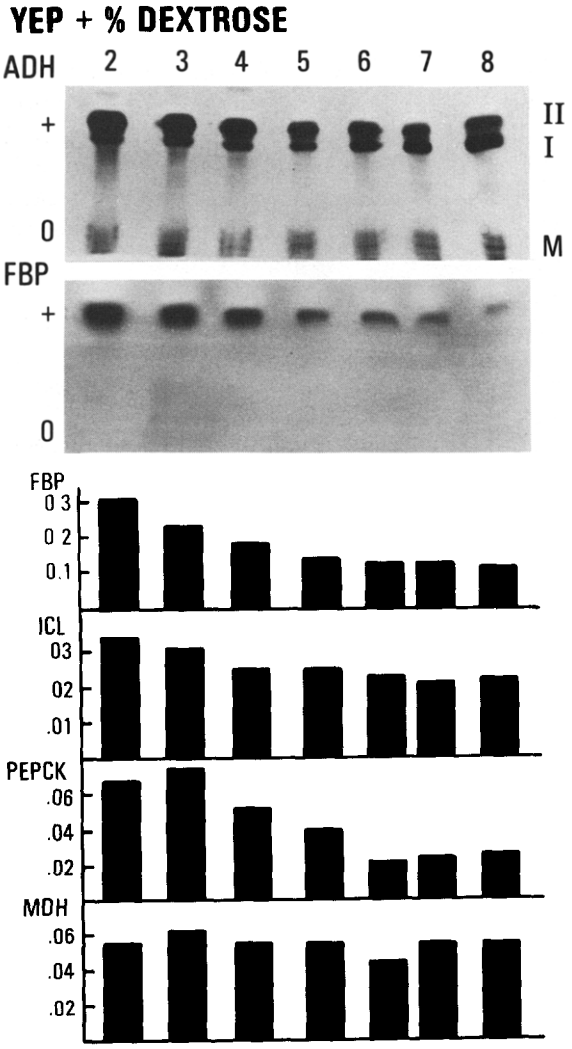

Fig.2. Effect on enzymes of varying dextrose levels in complete medium. Legend as in fig.1.

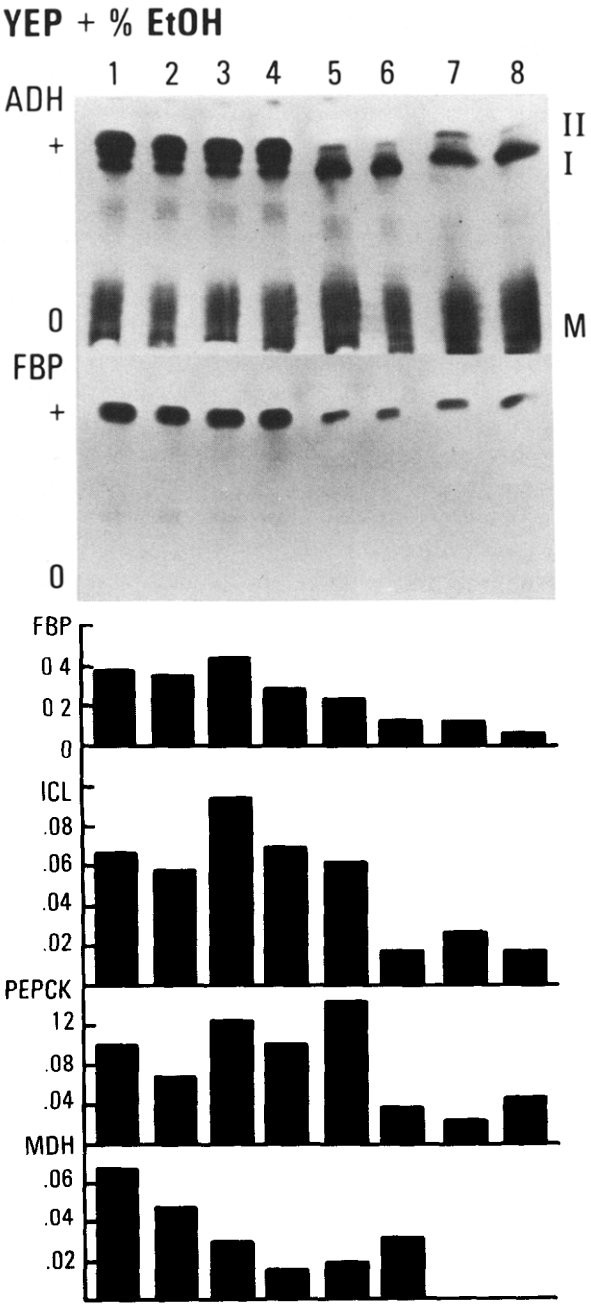

Fig.3. Effect on enzymes of varying ethanol levels in complete medium (ethanol as sole carbon source). Legend as in fig.1.

noticeably affected even by very high ethanol levels.

Beneath the gels in each figure is a series of histograms showing the spectrophotometrically determined levels of FBP, isocitrate lyase, malate dehydrogenase and phospho*enol*pyruvate carboxykinase found in each sample. These are expressed as μmol NAD reduced or oxidized/min per mg crude extract protein in the direct or the coupled assay. Some of the activity levels of the latter 3 enzymes are correlated with ADHII and FBP levels, while some are not. While total malate dehydrogenase activity declines markedly with increasing ethanol levels, it is essentially unaffected by alterations of dextrose or yeast extract levels. This assay does not distinguish the relative contributions of the cytoplasmic and mitochondrial isozymes. There is a decrease in isocitrate lyase activity at high levels of dextrose, ethanol and yeast extract, the last being in contrast to the effect of yeast extract on ADHII and FBP levels. Further, the activity of this enzyme is high on minimal medium with 0.1 or 0.3% yeast extract, conditions under which little ADHII or FBP are synthesized. The enzyme which most closely parallels the activities of ADHII and FBP is phospho*enol*pyruvate carboxykinase, and efforts are being made to construct an activity gel assay for this enzyme that will permit further investigation of this possible co-regulation. Further, high levels of ethanol have a repressive effect on all the enzymes tested except ADHI, which is surprising since its preferred direction is towards ethanol rather than acetaldehyde.

The activities of all these enzymes except for malate dehydrogenase are essentially zero when cells are grown in the presence of 1 ppm antimycin A, which converts glucose-grown cells into petite phenocopies (not shown).

Fig.4 shows two consecutive slices of the same gel showing a tetrad segregating for the *fbp* mutant. The first is stained for FBP activity, the second for ADH activity. It will be seen that the absence of FBP activity in the mutant has no effect on the synthesis of ADHII. Activity levels of the other 3 enzymes are in the normal range in the mutant.

A total of 20 spontaneous revertants of the *fbp* gene, able to grow on complete medium in the presence of a non-fermentable carbon source, were examined by horizontal starch gel electrophoresis and activity staining. No mobility mutants were detected, so that we currently have no evidence concerning the location of the *fbp* lesion. It may be in the structural gene itself, in a regulatory region linked to the structural gene, or in a regulatory gene unlinked to the structural gene (analogous to the *adr1* mutants of Ciriacy [13]).

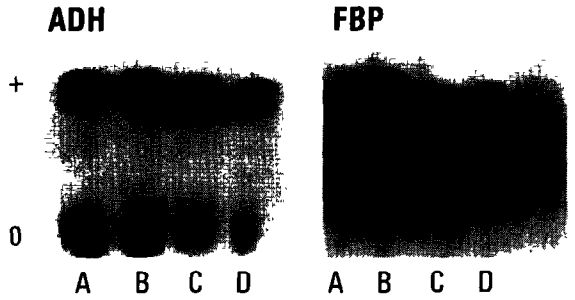

Fig.4. Activity stain of a tetrad segregating for the *fbp* mutant, grown to plateau on complete medium. Segregants A and C lack the enzyme. Note the lack of effect of the *fbp* mutation on ADHII activity.

3.2. *The mutational lesion* fbp *and the structural gene for ADHII are unlinked*

A strain carrying the *fbp* gene was crossed to the ADH-negative strain XW942-1B. Of a total of 10 tetrads examined by electrophoresis and replica plating, there were 6 tetratypes, two parental ditypes and two non-parental ditypes with respect to *FBP* and *ADR2*, the structural gene for ADHII. There was therefore no indication of linkage between these two genes.

4. DISCUSSION

The activity gels presented here show that ADHII and FBP are coordinately repressed when cells are grown to plateau on media with a high ratio of glucose or ethanol to cell extract components in the medium. Since we have shown [12] that the regulation of ADHII is not directly correlated with the level of glucose in the medium – the enzyme appears before the depletion of glucose in complete medium and does not appear even after the depletion of glucose on minimal medium – it is apparent that the coordinate regulation of these two enzymes is more complex than a simple response to glucose levels in the medium.

A mutant lacking the FBP activity was recently reported by Gancedo and Delgado [14]. It was found by screening for mutants able to grow on gluconolactone but not ethanol, an approach different from the one taken here. No evidence was presented concerning whether the mutation was in the structural gene. The deficiency in fructose-1,6-bisphosphatase was shown by a spectrophotometric assay.

We have presented the regulation data for ADHII in the form of activity gels alone because spectrophotometric data would be unable to distinguish between the activities of the 3 ADH isozymes. The spectrophotometric assay, however, does indicate that the activity levels of phospho*enol*pyruvate carboxykinase may be co-regulated with those of the other two enzymes. Malate dehydrogenase and isocitrate lyase activities do not appear to be co-regulated with this enzyme family, although without good gel assays for these enzyme activities it is not possible to determine whether isozymes of these enzymes are in fact co-regulated. We have recently developed a gel assay for visualizing all the isozymes of malate dehydrogenase in yeast [15], but it requires the clean separation of cytoplasmic and mitochondrial fractions.

The regulation of alcohol dehydrogenase II has been shown by Denis et al. [16] to be at the level of transcription. These workers also showed that a constitutive mutation at the unlinked positive regulatory locus *ADR1* resulted in the production of 5–6-times as much ADHII mRNA in the mutant as in the wild type grown under the same conditions. This suggests that there may be more than one regulatory mechanism operating at this locus.

In this regard it is of interest to note that the carbon catabolite repression mutants (*ccr* of Ciriacy [17]) markedly repess the levels of ADHII, FBP and other enzymes in aerobically grown cells, and will continue to do so even in the presence of the unlinked constitutive regulatory gene $ADR1^c$, which normally derepresses ADHII even in a petite cell. This last gene, however, has no effect on FBP. It therefore seems possible that the co-repression of these two enzymes by *ccr* mutants is in the nature of a 'fine-tuning' of the enzyme levels after they have been released from the repression seen in petite cells. Different enzymes may be repressed in petites by different mechanisms, which would explain why the *FBP* gene is unaffected by *ADR1* and why no mutant has yet been found that derepresses both ADHII and FBP in petite cells.

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REFERENCES

[1] Fraenkel, D.G. (1982) in: The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression (Strathern, J.N. et al. eds) pp.1–37, Cold Spring Harbor, NY.
[2] Wills, C. (1976) Nature 261, 26–29.
[3] Duntze, W., Neumann, D., Gancedo, J.M., Atzpodien, W. and Holzer, H. (1969) Eur. J. Biochem. 10, 83–89.
[4] Wills, C. and Melham, T. (1984) Arch. Biochem. Biophys., in press.
[5] Ciriacy, M., unpublished.
[6] Wills, C. and Phelps, J. (1975) Arch. Biochem. Biophys. 167, 627–637.
[7] Fink, G.R. (1970) Methods Enzymol. 17A, 59–78.
[8] Dixon, G.H. and Kornberg, H.L. (1959) Biochem. J. 72, 3P.
[9] Polakis, E.S. and Bartley, W. (1965) Biochem. J. 97, 284–297.
[10] Hanson, R.J., Hintze, H. and Holzer, H. (1976) Anal. Biochem. 74, 576–584.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Wills, C. and Martin, T. (1984) Biochim. Biophys. Acta 782, 274–284.
[13] Ciriacy, M. (1976) Mol. Gen. Genet. 154, 213–220.
[14] Gancedo, C. and Delgado, M.A. (1984) Eur. J. Biochem. 139, 651–655.
[15] Wills, C. and Martin, T. (1984) submitted.
[16] Denis, C.L., Ciriacy, M. and Young, E.T. (1981) J. Mol. Biol. 148, 355–368.
[17] Ciriacy, M. (1979) Mol. Gen. Genet. 176, 427–431.