ALTERATION IN THE REDOX BALANCE OF YEAST LEADS TO ALLYL ALCOHOL RESISTANCE

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1. Introduction

Work done in this laboratory has led to the conclusion that alterations in the ratio of NAD to NADH inside the yeast cell can confer resistance to allyl alcohol [1]. This is because a reduction in this ratio shifts the equilibrium amounts of allyl alcohol and its poisonous aldehyde acrolein in the direction of the harmless alcohol. Mutant enzymes of yeast alcohol dehydrogenase have been selected for allyl alcohol resistance under conditions in which alcohol dehydrogenase activity is required for survival. These mutants apparently produce such shifts. They have been characterized both kinetically [1,2] and at the amino acid level [3]. Here, we show first that when alcohol dehydrogenase is not present, exogenous concentrations of ethanol and acetaldehyde which normally produce shifts in the ratio fail to do so, and second that a wild-type enzyme that alters the ratio in the same direction as the mutant enzymes also confers a similar degree of allyl alcohol resistance. Thus, the ratio is indeed coupled to allyl alcohol resistance when a functioning enzyme is present.

The two major isozymes of yeast (*Saccharomyces cerevisiae*) alcohol dehydrogenase are found in the cytoplasm. The constitutive isozyme ADH-I, coded by the gene *ADC* [4], appears to be primarily responsible for ethanol production and the inducible isozyme ADH-II (coded for by *ADR2*) [4] is responsible for the first step in ethanol utilization under aerobic conditions. Normally, *ADR2* is repressed both under anaerobic conditions and when the cell is petite (unable to respire aerobically, even in the presence of oxygen). Further, a cell which is a petite or a petite phenocopy or is grown under anaerobic conditions, cannot survive unless it exhibits cytoplasmic alcohol dehydrogenase activity [5].

This last property was used to select for petite strains resistant to allyl alcohol. In petite cells, almost half the resistant mutations could be traded to an alteration in the electrophoretic mobility of the alcohol dehydrogenase. Since neither allyl alcohol nor acrolein is metabolized further by the cell [2], it was hypothesized that the resistance was due to a shift in the ratio NAD to NADH in the cell; a decrease in this ratio would result in the equilibrium between allyl alcohol and acrolein being driven in the direction of the harmless alcohol.

Considerable circumstantial evidence supports this view. Direct measurements of NAD and NADH showed a smaller ratio in mutant than in wild-type cells [1,2]. Addition of ethanol to the medium shifted the NAD to NADH ratio more markedly in the mutant cells than in the wild-type, and enhanced the resistance of mutant cells. Addition of acetaldehyde lowered the resistance [2]. Kinetic changes in the mutant enzymes can be interpreted as leading to these shifts. Yet two questions remain: (1) are these shifts due entirely to the action of the ADH; (2) are they large enough to produce the observed resistance?

2. Materials and methods

To examine these questions, we determine the amounts of NAD and NADH and their ratios in 4 classes of mutant:

(i) A wild-type petite, XW517-2D: This strain is sensitive to 0.5 mM allyl alcohol in the absence of ethanol in the medium, and to 2 mM allyl alcohol in the presence of 2% (v/v) ethanol.

(ii) A typical electrophoretic mobility mutant, CII-40-80E, α *ADE1*, selected in the turbidostat to be resistant to 20 mM allyl alcohol in the presence

of 2% ethanol and resistant to 4 mM in its absence.

(iii) Two ADH-negative strains, selected by repeated allyl alcohol selection using the technique pioneered in [6]. XW100-22A, *α adc adr2 adm his4*, an outcross of a multiple mutant derived by M. Ciriacy, is resistant to 300 mM allyl alcohol and its resistance is unaffected by the presence of ethanol or acetaldehyde in the medium. This strain shows no alcohol dehydrogenase activity even after prolonged culture at plateau. XW26, *α adc adr2 adm adel*, was derived independently in this laboratory and is also resistant to 300 mM allyl alcohol. However, its resistance is slightly reduced to 200 mM in the presence of 0.04% acetaldehyde. This strain produces limited amounts of ADH-II after some days; the level after 6 days at plateau is 3% that of the wild-type cells.

(iv) A strain derived by M. Ciriacy [7] in which the ADH-II structural gene has been derepressed by a mutation in the unlinked regulatory gene *ADR1*. This strain lacks ADH-I and the mitochondrial enzyme, and its designation and genotype are R234.1–9A, *α ura3 his4 trp2 adc 1–11 adm ADR1*-5C *ADR2*-F. The strain continues to produce ADH-II even when it is converted to a petite.

The strain was obtained by Ciriacy through scoring a large number of revertants of an *adc adm* ADR2 strain which were capable of survival on 8% glucose in the presence of 1 ppm of antimycin A. This inhibitor, which affects both the NADH and succinate branches of the electron-transport chain, converts grande cells into petite phenocopies. Normal *ADR2* strains would be expected to die in the presence of the inhibitor when grown at high glucose, since ADH-II would be repressed. While many of the mutants represented back mutations to ADH-I activity, some including the present strain grew because ADH-II activity had been derepressed. The derepressed ADH-II enzyme is wild-type, since the *ADR2* structural gene has not been subject to allyl alcohol selection.

Growth was on standard yeast extract–peptone–dextrose medium with the addition of metabolites and allyl alcohol to the levels indicated. All cell suspensions were diluted to $1.25–2.2 \times 10^7$ cells/ml in fresh medium and grown for 3 h before analysis. The initial and final cell concentrations are given in table 1, and it will be seen that the different media had no consistent effect on growth rate. The cells were grown in the presence of oxygen. NAD and NADH were measured by modification of the cycling assay in [8]. Details of the assay have been presented in [2], but the accuracy of the assay has since been improved. Pyridine nucleotides were extracted rapidly from log phase cells by hot 50% acid or alkaline ethanol. Then oxaloacetate, alcohol dehydrogenase and malate dehydrogenase were added to the extract. Care was taken to ensure that the cycling enzymes were free of endogenous pyridine nucleotide. Cycling results in the buildup of malate from the oxaloacetate. After 1 h cycling, the ADH, MDH and excess oxaloacetate were destroyed by heating. The malate was then measured by malic enzyme, which used NADP as a cofactor. Here, all media were filter-sterilized rather than autoclaved to reduce background absorbance. The cycling enzymes ADH and MDH were dialyzed for 12 h against 2 changes of 100 mM KPO_4 (pH 7.0) to remove unbound endogenous cofactor. As much precipitate as possible was removed by centrifugation after each step. Finally, it was found that the pH of the cycling mix tended to drift after the addition of the sample in the break solution. This was corrected by adjusting the pH as cycling commenced. All these corrections resulted in consistently higher values for NAD and particularly for NADH than were reported in [2]. Since the conclusions drawn in [2] and here depend on the value of the ratio of NAD to NADH, however, they remain unaffected.

All measurements were made on a series of 4 concentrations of cell extract, and the concentration of NAD or NADH in the cells was determined by fitting a linear regression line to the points. The standard errors derived from the regression are shown. Standard errors on the ratio of NAD to NADH were calculated using an approximation based on the first term of a Taylor series expansion. If $\bar{x}$ and $\bar{y}$ are the average NAD and NADH values, then the variance of the ratio is approximated by:

$$\sigma_R^2 = \frac{1}{\bar{y}^2} \left(\sigma_x^2 + \sigma_y^2 \bar{x}^2/\bar{y}^2\right)$$

Most standard errors are 1–20% of the value of the ratio.

Cell dry weight was determined by resuspending the washed pellet from 1 ml cell suspension in 95% ethanol in a tared tube, drying slowly through evaporation at 30°C, then drying briefly in a desiccator at 40°C.

Table 1
NAD and NADH (μmoles/g dry weight of cells) and resistance to allyl alcohol of the six yeast strains in this study

Strain	ADH expressed	Medium	Initial cell conc. ($\times 10^7$)	Final cell conc. ($\times 10^7$)	NAD (μmol/g)	NADH	NAD/NADH	Resistant to allyl alcohol at (mM)
XW517-2D (petite)	ADH-I	Normal	1.5	3.3	0.62 ± 0.11	0.57 ± 0.10	1.09 ± 0.26	0.5
		+EtOH	1.5	2.7	0.65 ± 0.03	0.98 ± 0.02	0.66 ± 0.03	2
		+acet.	1.5	3.5	0.58 ± 0.09	0.39 ± 0.08	1.49 ± 0.38	0.5
CII-40-80E (petite)	ADH-I (mutant)	Normal	1.25	2.9	2.20 ± 0.17	3.28 ± 0.03	0.67 ± 0.05	4
		+EtOH	1.25	2.6	1.82 ± 0.24	4.04 ± 0.23	0.27 ± 0.06	20
		+acet.	1.25	2.3	3.02 ± 0.41	3.07 ± 0.27	0.98 ± 0.16	2
XW100-22A	None	Normal	2.2	3.8	2.22 ± 0.20	3.17 ± 0.29	0.70 ± 0.09	300
		+EtOH	2.2	3.7	2.36 ± 0.18	3.43 ± 0.65	0.69 ± 0.14	300
		+acet.	2.2	3.8	1.48 ± 0.04	2.92 ± 0.03	0.51 ± 0.01	300
XW26	Trace ADH-II in old cultures	Normal	1.25	3.9	3.24 ± 0.35	1.53 ± 0.03	2.11 ± 0.23	300
		+EtOH	1.25	3.9	3.00 ± 0.27	1.21 ± 0.06	2.48 ± 0.27	300
		+acet.	1.25	3.8	3.04 ± 0.05	1.13 ± 0.18	2.70 ± 0.43	200
R234.1–9A	ADH-II	Normal	1.25	3.8	2.34 ± 0.47	2.31 ± 0.42	1.01 ± 0.27	1
		+EtOH	1.25	3.3	0.56 ± 0.11	2.29 ± 0.21	0.25 ± 0.05	10
		+acet.	1.25	3.9	2.30 ± 0.09	1.52 ± 0.11	1.51 ± 0.12	0.5

3. Results

The results are shown in table 1. Measurements were made on cells grown in 3 different media: normal YEPD (yeast extract–peptone–dextrose), YEPD plus 2% ethanol, and YEPD plus 0.04% acetaldehyde (redistilled before use). The last column shows the allyl alcohol resistance level on the 3 media. This resistance was measured by growth on freshly poured plates consisting of the 3 media with varying amounts of allyl alcohol.

In all the strains in which an ADH is expressed, the resistance to allyl alcohol reflects the ratio of NAD to NADH. The lower the ratio, the greater the resistance. It is striking that R234.1–9A, the strain with a derepressed wild-type ADH-II, shows a ratio as small in the presence of ethanol as does CII-40-80E, the strain expressing the mutant ADH-I, and this strain also shows a high level of resistance to allyl alcohol on this medium. On normal medium, however, the ratio is higher in the derepressed ADH-II strain than in the strain carrying the mutant ADH-I, and the resistance is correspondingly lower (resistant to only 1 mM compared with 4 mM).

The kinetics of ADH-I and ADH-II lead to the expectation that a strain expressing the latter will be more allyl alcohol resistant than one expressing the former [1]. The apparent K_m values for ethanol at infinite cofactor concentration for the two isozymes are 2.4×10^{-2} and 2.7×10^{-3} M, respectively. Further, the positive cooperativity exhibited by ADH-I will result in the effective K_m under physiological conditions being even larger, and the negative cooperativity exhibited by ADH-II will result in an even smaller physiological K_m. The K_m values for NAD for the 2 isozymes are not greatly different from each other (2.4 and 1.4×10^{-4} M, respectively). Thus, if ethanol concentration inside the cell were low and both isozymes were present in equal concentration, the rate of conversion to acetaldehyde by ADH-II would be expected to be >10-times greater than the rate due to ADH-I at the same ethanol concentration.

This effect is reflected in the much higher ratio of NAD to NADH seen in XW517, in which ADH-I is expressed, than in the derepressed ADH-II strain when both are grown in the presence of ethanol. Obviously, since these are ratios taken from the cell as a whole, any compartmentalization might allow even more extreme ratios in the immediate environment of the reaction. Such an effect may account for the fact that the resistant ADH-I mutant is more resistant than the ADH-II derepressed strain in the presence of ethanol, even though the ratios of NAD to NADH are the same.

4. Discussion

The investigation of the ratios of NAD to NADH in the ADH-negative strains XW100-22A and XW26 demonstrates clearly that an endogenous ratio characteristic of the particular strain is present even in the absence of ADH activity, and that in the case of these 2 extremely resistant strains their resistance is due to an absence of ADH activity and not to a skewing of the ratio. Media which strongly affect the ratio in strains with ADH activity have no significant effect on the 2 ADH-negative strains, though the slight decrease in resistance shown on acetaldehyde by XW26 may be reflected in the slight increase in the ratio on that medium. This may in turn be due to the trace of ADH-II activity still present.

Each strain appears to have its own characteristic basal amounts of NAD and NADH, which seem to reflect differences in the background genotypes of the strains. Alcohol dehydrogenase acts to perturb these quantities, though if both isozymes are present the ratios are stabilized [2]. The actual amounts seen are of course a reflection of the entire metabolism of the cell. In strains with active enzyme, the disturbance in the ratio accounts for most if not all of the allyl alcohol resistance.

This demonstration that the kinetics of ADH isozymes can be used to predict allyl alcohol resistance explains the remarkable specificity of the allyl alcohol selection system, which leads to a very large proportion of allyl alcohol resistant mutations traceable to the *ADC* (ADH-I) structural gene [2].

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