¹³C NMR analysis of a developmental pathway mutation in *Saccharomyces cerevisiae* reveals a cell derepressed for succinate dehydrogenase

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¹³C nuclear magnetic resonance (NMR) spectroscopy was used to study the metabolism of [2-¹³C]acetate in a diploid strain of *Saccharomyces cerevisiae* homozygous for the *spo50* mutation. This mutation results in failure to initiate sporulation and suppresses *spd* mutations (which cause derepressed sporulation). By analysing the pattern of ¹³C-labelling in glutamate it was deduced that the glyoxylate cycle is responsible for most of the acetate utilization and that there is very little tricarboxylic acid cycle activity. The labelling of α,α' -trehalose indicated that gluconeogenesis and the hexose monophosphate pathway operate in a similar way to the wild-type. The mutant strain has higher levels of succinate dehydrogenase than the wild-type. All of the physiological alterations caused by the *spo50* mutation can be explained by this difference.

Introduction

Sporulation in diploid strains of *Saccharomyces cerevisiae* is a simple unicellular model of differentiation. The switch from vegetative growth and mitosis to the alternative developmental pathway involves meiosis, a relatively rare event that occurs only in cells of specialized tissues in higher eukaryotes. The process is attractive for study because it involves the coordination of a complex sequence of genetic, biochemical and morphological events. The developmental switch occurs only in the G1 phase of the cell cycle in cells that are heterozygous at the mating type locus and respiratorily complete. It is conveniently induced by transferring cells to acetate in the absence of nitrogen.

Searches for mutations which specifically affect sporulation have revealed four different classes of mutant: those in which meiosis is induced regardless of the information at the mating type locus (reviewed by Kassir & Simchen, 1989); those which are defective in some stage of the process and hence are unable to complete it (Esposito *et al.*, 1970; Esposito & Esposito, 1974; Esposito & Klapholtz, 1981); mutants derepressed for initiation of sporulation which sporulate in rich

nutrient conditions where the wild-type would continue vegetative proliferation (Dawes, 1975; Shilo *et al.*, 1978; Vezinhet *et al.*, 1979; Dawes & Calvert, 1984); and mutants which are unable to initiate sporulation and continue cell cycling when the wild-type would initiate sporulation (Calvert & Dawes, 1984).

We have studied the metabolic changes involved in the initiation and execution of sporulation (Dickinson, 1988). Upon initiation of sporulation in acetate there is a rapid change in the operation of the tricarboxylic acid (TCA) and glyoxylate cycles such that most of the acetate taken up is converted to glutamate (Dickinson *et al.*, 1983). This happens because the activity of 2-oxoglutarate dehydrogenase is reduced (Dickinson *et al.*, 1985). At about 4 h, net glutamate synthesis ceases and trehalose synthesis starts using intermediates derived mainly from the glyoxylate cycle. There is an absolute requirement for an intact sequence of gluconeogenic enzymes to accomplish this phase of the process (Dickinson & Williams, 1986). Subsequently, fatty acid synthesis begins, concomitant with the onset of operation of the hexose monophosphate pathway (HMP). Apparently, yeast diverts carbon around the HMP in order to generate the NADPH required for fatty acid biosynthesis (Dickinson & Hewlins, 1988). The physiological significance of the large amount of fatty acid biosynthesis is unknown because about 75% of it is not essential (Dickinson & Hewlins, 1988), but it has been suggested that lipid

Abbreviations: TCA cycle, tricarboxylic acid cycle; HMP, hexose monophosphate pathway.

vesicles are required for spore wall development (Esposito & Klapholz, 1981).

Because ^{13}C nuclear magnetic resonance (NMR) spectroscopy had been useful in earlier studies of sporulation for the analysis of metabolism in both wild-type and mutant strains (Dickinson *et al.*, 1983; Dickinson & Hewlins, 1988), it was decided to use it to try to discover the metabolic defect in a strain that fails to initiate sporulation. The strain chosen carries the *spo50* mutation, which was originally isolated as an extragenic suppressor of *spd1* and *spd3* mutations which cause derepressed sporulation (Calvert & Dawes, 1984). Hence the answer sought had to explain not only why a cell carrying an *spo50* mutation should fail to initiate sporulation, but also the biochemical basis of suppression of *spd* mutations by *spo50* mutations.

Methods

Strains, media and cultural conditions. Strain S41 (*MATa/MAT α HO/HO arg4-1/arg4-1 cyh1/cyh1*), which was originally obtained from H.O. Halvorson (Brandeis University, Waltham, Mass., USA) was used as the wild-type. Strain 69.10C (*MATa/MAT α HO/HO arg4-1/arg4-1 ura3/ura3 spo50-1/spo50-1*), homozygous for the *spo50-1* mutation, was described by Calvert & Dawes (1984). Apart from the specific mutations denoted the two strains are isogenic. Cells were grown at 30 °C in conical flasks filled to 40% nominal capacity. YEPD medium comprised (l^{-1}): 10 g yeast extract, 20 g bacteriological peptone, 20 g glucose, 0.1 g adenine and 0.1 g uracil. Cells raised in YEPD starter cultures were used to inoculate presporulation medium which comprised (l^{-1}): 10 g yeast extract, 20 g bacteriological peptone, 20 g potassium acetate, 1 g glucose, 0.1 g adenine and 0.1 g uracil. Sporulation medium comprised (l^{-1}): 10 g sodium acetate (sodium $[2-^{13}\text{C}]$ acetate for NMR experiments), 0.2 g adenine, 0.2 g uracil and 0.15 g benzyl penicillin in 100 mM-potassium chloride. Sporulation was induced by the method of Fast (1973).

Extraction of metabolites, NMR analysis and enzyme assays. Cells were harvested and extracts prepared for analysis using a Bruker WM360 spectrometer operating at 90.5 MHz as described previously (Dickinson & Hewlins, 1988). The major signals were assigned from known chemical shifts. Each consisted of a combination of multiplets corresponding to different isotopomers. These were identified following earlier work and the total intensity of each component was determined from expanded spectra by summation of the isotopomer intensities. After 12.5 h in sporulation medium cells were harvested by rapid filtration, washed and disrupted using glass beads as described previously (Dickinson & Williams, 1986). Succinate-cytochrome *c* oxidoreductase was assayed as described previously (Dickinson *et al.*, 1986). Malate dehydrogenase was assayed in the reverse direction (oxaloacetate to malate) by following the oxidation of NADH to NAD $^{+}$ at 340 nm. The reaction mixture comprised 900 μl 0.2 M-potassium phosphate buffer, pH 6.4, 100 μl 2% (w/v) sodium azide, 12 nmol NADH and 5 μl of appropriately diluted cell extract. This was preincubated for 3 min before initiating the assay by adding 25 nmol oxaloacetate.

Results and Discussion

Samples were removed from cultures at various times after transferring cells of strain 69.10C to sporulation

Fig. 1. ^{13}C NMR spectrum obtained at 90.5 MHz of a perchloric acid extract of strain 69.10C made 12 h after transfer to sporulation medium containing $[2-^{13}\text{C}]$ acetate. Tre C-1 to C-6 refer to the carbon atoms of α,α' -trehalose; Glu C-2 to C-4 refer to the carbons of glutamate; Cit denotes the C-1 and C-4 resonances of citrate and Gly represents the C-1 and C-3 resonances of glycerol.

medium. Microscopic examination of many thousands of cells revealed there was no sign of sporulation having been initiated even after 48 h. ^{13}C NMR spectra of perchloric acid extracts were obtained and a number of ^{13}C -labelled metabolites were identified (Fig. 1). It has been shown that the precise distribution of label in an intermediate ('isotopomer population') can be used to calculate the individual contribution of the different pathways that can give rise to that compound (reviewed by London, 1988). This is because an isolated ^{13}C nucleus gives a single resonance (singlet) unless attached to one or more ^{13}C nuclei, in which case various multiplets are produced, characteristic of the labelling pattern. Previously, we and others have selected certain key intermediates as reporters of the metabolic fluxes within different pathways (den Hollander *et al.*, 1979, 1981; Walker *et al.*, 1982; Dickinson *et al.*, 1983; Dickinson & Hewlins, 1988). The compounds of relevance to this study are glutamate (the isotopomers of which reveal the relative rates of the TCA and glyoxylate cycles) and trehalose which yields information on gluconeogenesis and the HMP.

A variety of overlapping multiplets was observed (Fig. 1) for each glutamate carbon from which the intensity due to each isotopomer was determined. When analysing the ^{13}C multiplets derived from glutamate the known biochemical pathways were always borne in mind. Hence, a singlet C-4 could arise only from $[4-^{13}\text{C}]$ glutamate or from $[2,4-^{13}\text{C}]$ glutamate, since no other isoto-

Fig. 2. Proportions of ^{13}C -labelled glutamate species in strain 69.10C as a function of time. The species shown are the 2,3,4- (●), 2,4- (■), 1,3,4- (□), 1,2,3,4- (○) and 4- (▲) ^{13}C -labelled isotopomers.

pomer can be formed in which C-4 is not adjacent to another ^{13}C nucleus. The singlet at C-2 can arise only from 2,4- ^{13}C -labelled molecules since C-2 labelling alone cannot occur by known biochemical routes. Hence the amount of [2,4- ^{13}C]glutamate is proportional to the intensity of the C-2 singlet, and the amount of [4- ^{13}C]glutamate is proportional to the intensity of the C-4 singlet less the intensity of the C-2 singlet. Similarly, the amount of [1,3,4- ^{13}C]glutamate is proportional to the intensity of the C-1 singlet; other conceivable isotopomers, such as [1,3- ^{13}C]glutamate, which would give a singlet, are not biosynthetically possible. The amount of [1,2,3,4- ^{13}C]glutamate is proportional to the intensity of the C-1 doublet. The amount of [2,3,4- ^{13}C]glutamate is proportional to the intensity of the C-2 doublet. The amount of [3,4- ^{13}C]glutamate is proportional to the intensity of the C-4 doublet less the intensity of the C-2 doublet. Using these principles the amounts of each glutamate isotopomer at each time were determined from the signal intensities. The results are shown in Fig. 2.

A comparison of the proportions of different ^{13}C -labelled glutamate isotopomers with time shows that the 2,3,4-labelled species quickly rises to a maximum and thereafter remains the most abundant species. In contrast, the 1,2,3,4-labelled species is present only as a very small proportion. From this one may conclude that the glyoxylate cycle is responsible for most of the utilization of ^{13}C -labelled acetate and that there is very little contribution by the TCA cycle. The explanation for this is as follows: condensation of [2- ^{13}C]acetyl-CoA with oxaloacetate would give rise via the first turn of the TCA cycle to 2-oxoglutarate and hence glutamate labelled solely at C-4. Cycling of 2-[4- ^{13}C]oxoglutarate through the TCA cycle would give rise to [2,4- ^{13}C] and [3,4- ^{13}C]glutamate and further cycling of the doubly enriched species would give rise successively to [2,4-],

[1,3,4-] and [2,3,4-], and thence to [2,4-], [1,3,4-], [2,3,4-] and [1,2,3,4- ^{13}C]glutamate. A similar consideration of the glyoxylate cycle shows that glutamate would become labelled at the 4-, the 3,4- and the 2,3,4-positions. Hence [1,2,3,4- ^{13}C]glutamate can arise only from the operation of the TCA cycle. Thus, the very low proportion of this species indicates that there is very little TCA cycle activity. This is very different from what was found for the sporulation-competent parental strain S41 where, even though the activity of 2-oxoglutarate dehydrogenase is greatly reduced, both TCA and glyoxylate cycles are operating (Dickinson *et al.*, 1983).

Although some of the multiplets for trehalose are complex (e.g. the portion of the spectrum occupied by C-3, C-5, C-2 and C-4 of this molecule), we needed to analyse only the simpler resonances of C-1 and C-6, which are each composed of a singlet surrounded by the corresponding doublets (Fig. 1). The singlet C-1 resonance is due to labelling at C-1. The doublet C-1 of trehalose is due to additional labelling of the adjacent C-2. Similarly, the singlet C-6 resonance is due to labelling at C-6 and the intensity of the C-6 doublet is a measure of the amount of [5,6- ^{13}C]trehalose. Trehalose synthesis started at about 4 h (Fig. 3), i.e. with similar kinetics to that in the wild-type. Comparison of the doublet/singlet ratios for C-1,1' and C-6, 6' of trehalose with time also shows a similar pattern to the wild-type (Fig. 4; Dickinson *et al.*, 1983). From these two observations, we conclude there is normal gluconeogenesis and that the HMP is also functioning. This can also be understood by a consideration of the NMR phenomenon in relation to the metabolic pathways involved. Trehalose is made via gluconeogenesis from phosphoenolpyruvate which, in turn, comes from oxaloacetate. As explained above, in this strain under these conditions the oxaloacetate is derived almost exclusively from the glyoxylate cycle. Hence, phosphoenolpyruvate will be labelled at C-2 and C-3. This will give rise via gluconeogenesis to similar isotopic enrichments in both the C-1,2 and C-6,5 portions of glucose 6-phosphate and hence also in trehalose; this is because glucose atoms 1–6 are derived from phosphoenolpyruvate atoms 3,2,1; 1,2,3, respectively. If there is intervention of the HMP there is a significant change in the doublet/singlet ratio for C-1 in glucose 6-phosphate (and hence trehalose) because C-1 and C-2 in glucose 6-phosphate after cycling through the HMP are derived from C-2 and C-3, respectively, in the original glucose 6-phosphate molecules (Dickinson *et al.*, 1983; Dickinson & Hewlins, 1988).

From the NMR analysis it appeared that the asporogenous strain 69.10C (which is homozygous for the *spo50* mutation) was metabolically very like the wild-type strain except that the glyoxylate cycle was almost exclusively responsible for acetate utilization. The aim of

Fig. 3

Fig. 3. Synthesis of trehalose in strain 69.10C after transfer to sporulation medium containing [2-¹³C]acetate. The proportions of trehalose labelled 5,6-¹³C (■) and 6-¹³C (○) are shown.

Fig. 4

Fig. 4. Doublet/singlet ratios for the C-6,6' (○) and C-1,1' (●) resonances of α,α'-trehalose from strain 69.10C.

this work was to discover the biochemical reason for asporogony and suppression of *spd* mutations conferred by the *spo50* mutation. Two other facts had to be taken into account. Firstly, it was known that strains bearing *spd1* and *spd4* mutations all have reduced activities of 2-oxoglutarate dehydrogenase; the enzyme in these strains has both a larger K_m and a smaller V (Dickinson *et al.*, 1985). Secondly, strains carrying *spo50*, *spo51* or *spo53* mutations all show a constitutive ability to reduce tetrazolium (Calvert & Dawes, 1984). Tetrazolium is not reduced by the wild-type on glucose-containing media but is on glycerol. In contrast, strains bearing *spd* mutations cannot reduce tetrazolium under any conditions. These results were always taken to signify that *spd* mutations lead to the loss of a (previously unspecified) dehydrogenase (now thought to be 2-oxoglutarate dehydrogenase), and that the aforementioned *spo* mutations lead to the derepression of another (unspecified) dehydrogenase. The only way to account for all of these observations would be to posit that strain 69.10C is derepressed for either succinate dehydrogenase or malate dehydrogenase. Measurement of these two enzyme activities showed that the mutant strain 69.10C had almost threefold higher levels of succinate dehydrogenase (Table 1). On the other hand, the specific activity of malate dehydrogenase was virtually identical in both strains.

These results suggest that the *spo50* mutation leads to derepression of succinate dehydrogenase. At the moment, nothing more is known about the genetic details. For instance, the mutation has not been mapped to a specific chromosomal location and it cannot be discerned from this type of analysis whether any part of a structural gene, its promoter or upstream activation sequence are involved, or whether the mutation is in a

separate regulatory gene. Nevertheless, this result is useful for two reasons. Firstly, as far as the authors are aware, this is the first time that NMR has been used to discover the biochemical basis of a mutation affecting development. Secondly, and more important, it provides a complete explanation for the mode of action of the *spo50* mutation. When combined in a cell with an *spd* mutation (which reduces 2-oxoglutarate dehydrogenase activity and so switches the cell into a sporulation mode of metabolism) it will tend to increase the flow of carbon through the TCA cycle by ensuring that the small amount of succinate that is produced is rapidly converted into fumarate. This will counteract the effect of an *spd* mutation. A cell which is derepressed for succinate dehydrogenase would also give the tetrazolium reduction profile observed for these *spo* mutants. A strain which is homozygous for the *spo50* mutation (but carries no *spd* mutation) would be expected to route most of its carbon *via* the glyoxylate cycle when transferred to sporulation medium. This is because the increased conversion of succinate by succinate dehydrogenase will serve to accelerate the flux through the glyoxylate cycle by consuming the succinate produced by isocitrate lyase.

The NMR study produced one further surprise. This was the production of glycerol which rose to a maximum at 12 h (Fig. 1) and then declined. Glycerol has not been detected under these conditions as a metabolic product of any of the strains examined to date. It is usually observed in the early stages of a fermentation of sugar to ethanol. The generally held view is that glycerol is produced at such time so that the yeast cell can balance its pyridine nucleotide pool. A strain with elevated levels of succinate dehydrogenase will produce more NADH than the wild-type. One would expect that this would be oxidized via the electron transport chain. However, if the oxygen

Table 1. *Specific activities of malate dehydrogenase and succinate dehydrogenase in the wild-type and mutant strains*

Cells were harvested, washed and broken 12.5 h after transfer to sporulation medium. Aliquots of the diluted cell preparations were used to initiate the enzyme assays. The results are the means of five determinations $\pm$ SEM.

Strain	Relevant genotype	Malate dehydrogenase specific activity [nmol min ⁻¹ (mg protein) ⁻¹]	Succinate dehydrogenase specific activity [ΔA_{550} min ⁻¹ (mg protein) ⁻¹]
S41	Wild-type	160 $\pm$ 10	55 $\pm$ 3.6
69.10C	<i>spo50/spo50</i>	161 $\pm$ 11	158 $\pm$ 1.7

transfer rate into the culture was insufficient, NAD⁺ would become depleted. It may be that in this instance glycerol is produced as a by-product to enable the regeneration of NAD⁺.

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