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EFFECT OF MUTANTS AND INHIBITORS ON MITOCHONDRIAL TRANSPORT SYSTEMS IN VIVO IN YEAST

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We have reported elsewhere (Wills, C. and Martin, T. (1984) Biochim. Biophys. Acta 782, 274–284) that one or more mitochondrial transport systems may be involved in the regulation of the inducible alcohol dehydrogenase of yeast, ADH-II. In order to investigate this phenomenon further, it was necessary to determine which of these systems operate in the cell in vivo. We give in this paper preliminary evidence that inhibitors of the malate-phosphate (*n*-butyl malonate), malate-citrate (hydroxycitrate) and malate-α-keto-glutarate (aminooxyacetate or cycloserine) transport systems all operate in vivo. While the demonstration of the in vivo inhibitory activity of *n*-butyl malonate and hydroxycitrate is entirely by physiological methods, that of the transaminase inhibitors aminooxyacetate and cycloserine depends in part on the isolation of mutants capable of growth on glycerol in minimal medium. On this medium these mutants depend on the malate-aspartate shuttle for growth, and as expected the transaminase inhibitors prevent their growth. Two of the mutants show an enhanced rate of mitochondrial glutamate uptake. A preliminary survey of the properties of the glycerol growth mutants is presented, showing that the probable mode of action of these mutants is an increase in the efficiency of the malate-aspartate shuttle.

Introduction

We have found [1] that malate, glutamate and aspartate all produce strong induction and oxaloacetate a strong inhibition of alcohol dehydrogenase II, the enzyme chiefly responsible for the oxidation of ethanol. These compound are involved in some of the mitochondrial transport systems which transport reducing equivalents across the inner mitochondrial membrane in the form of malate. In order to investigate the role of these transport systems further, it was necessary to determine which of them are actually operating in vivo. Our laboratory has shown [2,3] that mutants lacking cytoplasmic alcohol dehydrogenase are absolutely dependent on the operation of the α-glycerol phosphate-dehydroxyacetone phosphate shuttle for growth. Studies on the in vivo activity of mitochondrial anion transport has been actively pursued in other cell systems (see Ref. 4 for review).

We show here, by a combination of studies with mutants and with inhibitors, that other transport systems also operate in yeast in vivo. Our strongest evidence concerns the malate-aspartate shuttle, with less certain information about the malate-citrate shuttle and malate-phosphate transport. Fig. 1a shows mitochondrial shuttle mechanisms suggested to be responsible for the transfer of reducing equivalents through the mitochondrial membrane in the form of malate. Malate is then oxidized with the concomitant reduction of NAD^+ to yield NADH. Fig. 1b shows the two major pathways by which reducing equivalents are transferred out of the mitochondrion by pathways leading to gluconeogenesis.

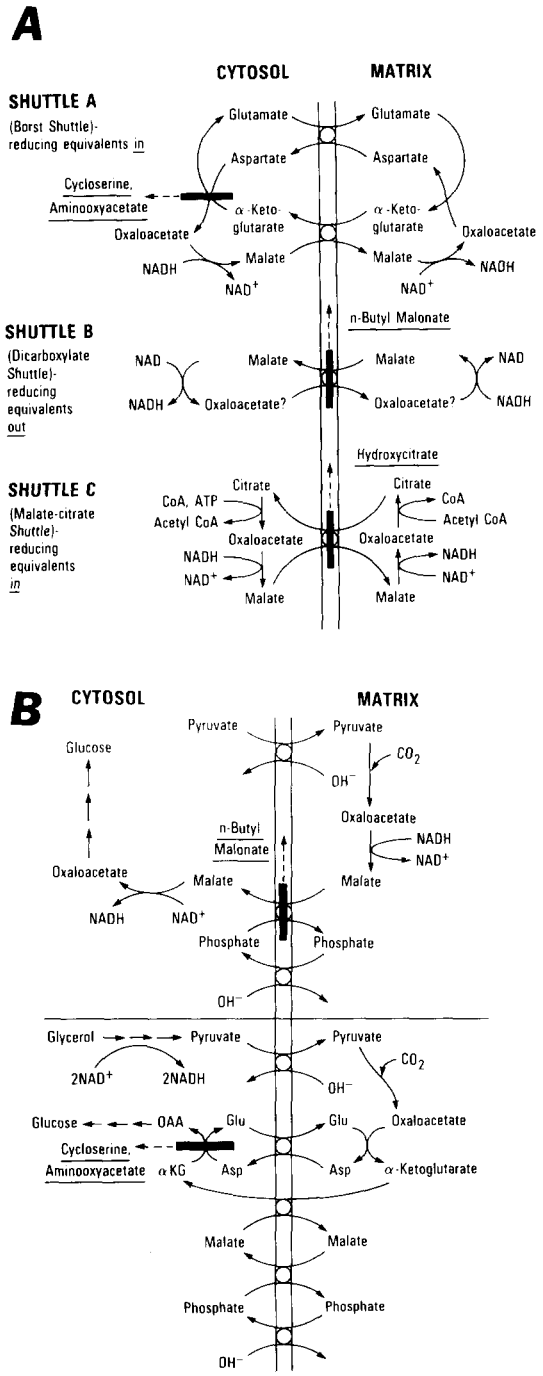

Fig. 1. (A) Shuttles transferring reducing equivalents across the mitochondrial membrane in the form of malate. Inhibitors of the shuttles are underlined, and their sites of action indicated by heavy bars. (B) Modified from Tzagoloff, 1982 [10]; based on Williamson, 1976 [9]. The upper diagram shows the probable course of gluconeogenesis when cytoplasmic NADH can be generated only by the release of malate from the mitochondria. The lower diagram shows the course of gluconeogenesis when cytoplasmic NADH is being generated by the catabolism of a non-fermentable substrate such as glycerol. Inhibitors of these pathways are underlined, and their sites of inhibition shown by heavy bars.

The first of the systems in Fig. 1a is the well-known Borst or malate-aspartate shuttle [5]. The second has been hypothesized [6] as a mechanism by which reducing equivalents could be transported from the mitochondrion to the cytoplasm, but it has not been demonstrated in yeast. It requires that the mitochondrial inner membrane be permeable to oxaloacetate. Further, since this shuttle is at least in theory bidirectional, it is not obvious why it should be operating in the cell if the Borst shuttle (the complexity of which is a necessary consequence of the impermeability of the inner membrane to oxaloacetate) is highly active in the cell. The third shuttle in the figure, the citrate-malate shuttle [7], is important in fatty acid catabolism and can be inhibited by citrate analogues.

Two more pathways concerned with the transfer of reducing equivalents across the mitochondrial membrane are shown in Fig. 1b. Both are gluconeogenesis pathways [8–10] and, rather than being shuttles in which the intermediates are continuously renewable, they are responsible for the one-way transfer of reducing equivalents out of the mitochondrion. These equivalents can subsequently be used in anabolic reactions. One of these latter pathways involves transamination and the other does not, so it has been possible to deal with the effects of these pathways individually by the appropriate use of inhibitors.

Materials and Methods

Yeast strains. Strains employed are listed in Table I.

Media. Complete media consisted of yeast extract (1% w/v) and Bacto-peptone (2% (w/v) (YEP), both from Difco, with the addition of dextrose (D), glycerol (G), sodium lactate (L), ethanol (E) or potassium acetate (Ac) as carbon sources (2% v/v or w/v). Minimal medium consisted of 0.75% (w/v) Difco yeast nitrogen base

TABLE I

STRAINS OF *SACCHAROMYCES CEREVISIAE* USED IN THIS PAPER

Strain	Genotypes	Source
Wild type		
X2180-1A	MATa SUC2 mal gal 2 Cup1	R.K. Mortimer
X2180-1B	MATα SUC2 mal gal 2 CUP1	R.K. Mortimer
X2180-1A-P5	ρ^- derived from X2180-1A	R.K. Mortimer
ADH mutant		
XW517-2D	MATa adc 1-11 ADR1 ADR2 adm	M. Ciriacy
Marker strains		
XW208-1A	MATa adel ade2	Derived from Z506 of Benjamin Dorfman
XW208-3B	MATα adel ade2	Derived from Z506 of Benjamin Dorfman

without amino acids (MV) with addition of the same set of carbon sources. Amino acid supplements to the minimal medium were added to a final concentration of 0.003%.

Inhibitors. A variety of known inhibitors of functions directly or indirectly involved in mitochondrial transport were used. All were added to the medium from sterile filtered stock solutions prepared immediately before use. Aminooxyacetate, *N*-ethylmaleimide and cycloserine were obtained from Sigma, *n*-butyl malonate from Aldrich, and (−)-hydroxycitrate as a kind gift from Professor John Lowenstein. When dry, the hydroxycitrate is in the lactone form, and the free acid was prepared by heating an aqueous solution of the lactone in the presence of 3 equivalents of NaOH at 90°C for 2 h.

Isolation and genetic analysis of mutants. Two general classes of mutants were obtained. The first, *glg* mutants, were derived from wild-type or adenine auxotroph cells, unmutagenized, plated on MVG or MVG with adenine, at a density of 10^7 cells/plate. After one week of incubation at 30°C, a small minority of the plated cells produced a lawn of microcolonies and a few larger colonies (three or four per plate) appeared. These larger colonies were picked, named *glg* (for glycerol growth), and investigated further.

The same non-mutant starting strains were used to select for mutants that could grow on MVG + 0.003% monosodium glutamate. Again, after one week of growth a large number of microcolonies was seen on a heavy background of nongrowing cells, but at an initial density of 10^7 cells/plate there were twenty or thirty large colonies and the mutants grew more rapidly than did the equivalent mutants isolated on MVG. These mutants were named *msg* mutants (for monosodium glutamate and glycerol growth), and investigated further. Genetic analysis was carried out by standard tetrad analysis.

Uptake of metabolites. ^{14}C-labeled glucose was purchased from ICN, (U-^{14}C)-labelled 3-*O*-methyl-D-glucose, glycerol, glutamate, malate and aspartate from Amersham and leucine from New England Nuclear. All were adjusted to 1 mM in the appropriate medium labelled to a level of 1 μCi/μmol, and the cells were adjusted to a final titer of $5 \cdot 10^8$ cells/ml. The incubated cell samples were rapidly collected by vacuum filtration. All experiments were run in duplicate and the results averaged. The filters were dried and counted in toluene with POPOP on a Beckman LS-230 liquid scintillation counter.

Isolation of mitochondrial proteins. Mitochondria were isolated by the method of Leon and Mahler [11]. Purity of the preparations was checked by activity-stained horizontal starch gel electrophoresis. The mitochondrial preparations showed only the mitochondrial alcohol dehydrogenase. Proteins were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue.

Mitochondrial uptake. Uptake of [^{14}C]glutamate by isolated mitochondria was determined at 30 s and 1 min by the methods of Meyer and Vignais [12]. Uptake was carried out in 0.9 ml aliquots to

which 0.1 ml aliquots of mitochondrial suspension were added. Uptake was terminated by centrifugation for 1 min in a Fisher microcentrifuge, and the supernatant was carefully removed by pipette. The pellet was resuspended in 1 ml of ice-cold 100 mM KCl, washed once, and resuspended in 1 ml sodium cholate (Sigma). The samples were counted in 5 ml of Monofluor (National Diagnostics).

Uptakes of ^3H_2O and [^{14}C]sucrose were also determined in order to obtain measures of the mitochondrial water content and the volume of the space between the inner and outer mitochondrial membranes. The concentrations of glutamate and sucrose were 1 mM, labelled at a level of 1 μCi/μmol. Tritiated water was labelled at a level of 1.5 μCi/ml. All experiments were run in duplicate and the results averaged.

Physiological studies. Measurements of glycerol consumed by wild type and *glg* cells were made with a Varian 2100 gas chromatograph (see Ref. 13 for details).

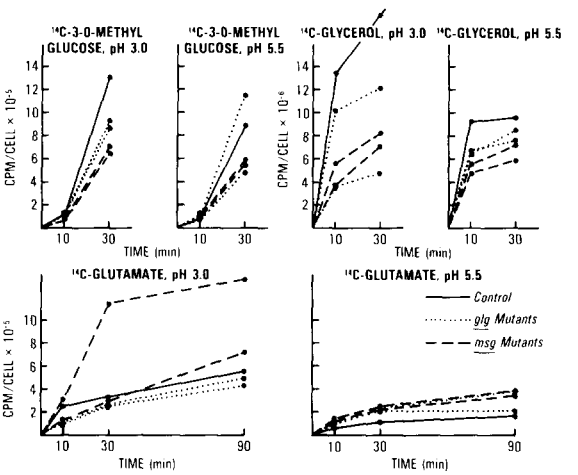

Fig. 2. Uptake of ^{14}C-labelled 3-*O*-methyl glucose, glycerol and glutamate by wild type cells and by *glg4*, *glg7*, *msg1* and *msg2* mutants. Uptake was carried out in 1 ml aliquots, with a metabolite concentration of 1 mM. Metabolites were labelled at a level of 1 μCi/μmol. Each point is the average of two experiments. The pH values refer to the pH of the medium.

Results

We began with the observation that wild type *Saccharomyces cerevisiae* is incapable of growth on minimal medium with glycerol as a sole carbon source (MVG). Yet, it is capable of utilizing glycerol when grown on complete medium with glycerol (YEPG). One possible reason for this discrepancy is the rate of transfer of reducing equivalents from the cytoplasm to the mitochondrial matrix. Mutants capable of growing on glycerol on minimal medium may have a mitochondrial transport system of increased effectiveness, either because the transport system is itself altered or because of an altered concentration of metabolites in the cell. Two fast growing mutants, capable of growing on MVG, designated *glg4* and *glg7*, were chosen for further investigation. Two others, designated *msg1* and *msg2*, were selected on MVG plus glutamate.

Physiological characterization of the wild type, glg and msg strains

(a) Metabolite uptake experiments. Cells were grown to log phase in minimal medium, then transferred to MV without a carbon source for the uptake experiments, which were carried out at pH 3.0 and 5.5. For 3-*O*-methylglucose and glycerol, no further changes were seen after 30 min of uptake.

Figs. 2 and 3 show the results. Both 3-*O*-methylglucose and glycerol were taken up slightly less efficiently by the mutant cells than by the wild type. One *msg* mutant, *msg1*, showed sharply increased uptake of glutamate at pH 3.0, and at pH

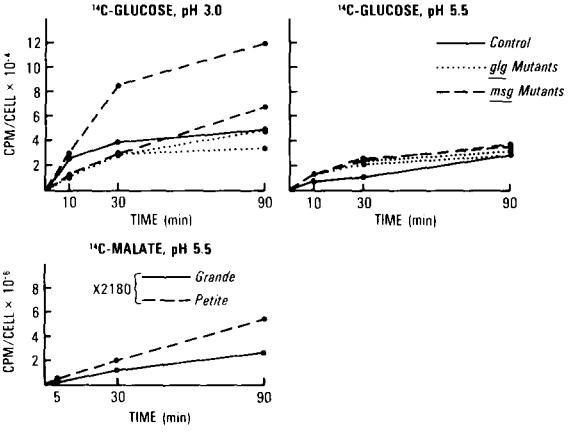

Fig. 3. Uptake of ^{14}C-labelled glucose and malate. In the case of malate, grande and petite wild-type strains were investigated, since it is unlikely that *glg* or *msg* mutants would have significantly altered malate uptake.

5.5 all the mutants showed a slightly increased uptake of glutamate over the wild type. The increased glutamate uptake is consistent with an increased ability of *msg1* to use glutamate as a sole carbon source (data not shown).

No growth of wild type is seen on MV plates with concentrations of sodium malate ranging from 1% to 10%, and it has proved impossible despite repeated attempts to select for mutants capable of growth. As with glycerol, however, permeability of the cell seems not to be the limiting factor, since malate is taken up slowly by both wild-type and petite cells.

(b) Glycerol utilization. Cells of the four mutants and wild type were grown on YEPG to plateau, 64 h after inoculation. Table II shows that the glycerol mutants varied greatly in their ability to utilize glycerol under these conditions, with the two *glg* mutants using about a quarter of the available glycerol and the two *msg* mutants using three quarters. The wild-type cells could only use one tenth of the available glycerol, but this is sufficient for them to grow much more rapidly on YEPG than on YEP medium.

Growth patterns on fermentable and non-fermentable media with various supplements and inhibitors

Fig. 4 shows the results of growth experiments performed in wild-type cells and the four representative *glg* and *msg* mutants. Growth of the wild-type strain is shown above the diagonal in each rectangle and that of the mutants below it. It will be seen that all the mutants showed the same growth pattern, and that this pattern was only different from that of the wild-type strain on MVG.

Analysis of the pattern seen in the figure can begin most easily with a consideration of the media involved. MVD, minimal medium with dextrose as a sole carbon source, is a medium on which the cells derive most of their energy from glycolysis. Gluconeogenesis therefore remains at a low level on this medium. While reducing equivalents are produced in the cytoplasm by glycolysis, NAD^+ can easily be regenerated in the cytoplasm by the operation of the glycerol phosphate-dihydroxyacetone phosphate shuttle.

The remaining media are all supplied with a non-fermentable carbon source. Glycerol, ethanol and lactate have in common that their metabolism generates reducing equivalents in the cytoplasm, reducing equivalents which must be transferred

TABLE II

GLYCEROL UTILIZATION OF WILD-TYPE, *glg* AND *msg* MUTANTS

The glycerol utilization is presented as a percent of the total when grown to plateau on YEPG (initial concentration of glycerol, 2% v/v).

Mutant	% glycerol utilized
X2180-1A	9.6
glg4	20.1
glg7	26.9
msg1	73.1
msg2	78.6

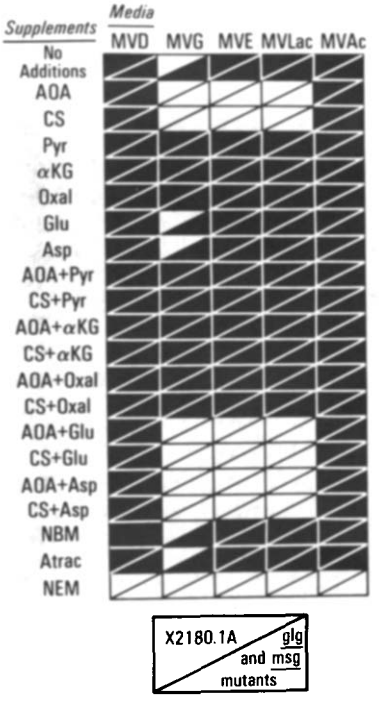

Fig. 4. Growth pattern seen with wild type, *glg* and *msg* mutants on a range of media. Black indicates growth, white indicates no growth. Abbreviations: (a) Inhibitors: AOA, aminooxyacetate, 0.5 mM; CS, cycloserine, 0.5 mM; NBM, *n*-butyl malonate, 5 mM; NEM, *N*-ethylmaleimide, 0.05 mM; Atrac, atractyloside, 5 mM. (b) Supplements (all added at 10 mM): Pyr, pyruvate; αKG, α-ketoglutarate; Oxal, oxaloacetate; Glu, glutamate; Asp, aspartate.

into the mitochondrion if cytoplasmic catabolism of these substrates is to continue. The growth data suggest that this is so even though lactate dehydrogenase activity in yeast is associated with cytochrome b_2 [14]. But when the cells are grown on the last medium in the table, MV with acetate as a sole carbon source, their growth is not dependent on this restriction. When grown on this medium, cells must transfer reducing equivalents out of the mitochondrion rather than into it. Thus, it is to be expected that growth on acetate medium should be qualitatively different from that on other non-fermentable media, and the figure bears this out.

The figure does not show all the supplements tried, but only those which are most germane to the present analysis. In addition to the listed compounds, all the remaining amino acids, and the remaining Krebs cycle, glyoxylate cycle, and urea cycle intermediates were tested, added to the media at 10 mM concentration. None of these supplements permitted wild-type cells to grow on MVG.

The effect of hydroxycitrate, an inhibitor of citrate lyase and therefore of the malate-citrate shuttle, was also examined. X2180-1A and *glg7* were grown on MVAc in the absence or presence of aminooxyacetate. Three different concentrations of hydroxycitrate, an inhibitor of the citrate-malate transporter, were added. Growth was inhibited in the presence of aminooxyacetate, but not in its absence. No inhibition was seen when the cells were grown on MVD. This experiment demonstrated both the effectiveness of hydroxycitrate as an inhibitor of citrate transport in vivo and the fact that transport systems involving transamination can continue to permit the cells to grow even when citrate transport is inhibited.

Evidence for the inhibition of growth by *n*-butyl malonate in the presence of aminooxyacetate was also obtained. This inhibitor has been shown to be highly effective in halting glyconeogenesis in perfused rat liver cells [6]. A strong inhibitory effect on growth of wild type and *glg7* when the two compounds were added together was seen on ethanol, but not on pyruvate (data not shown). The results show a sharp threshold effect between 1 and 2 mM *n*-butyl malonate, for which we have as yet no explanation. Prevention of growth by the two inhibitors was also seen when the *glg* mutant were grown on MVG.

Further growth characteristics of yeast on MVG

It will be seen from Fig. 4 that growth of wild-type cells on MVG can be 'primed' by the addition of low levels of pyruvate, oxaloacetate or α-ketoglutarate. We use the term 'primed' to indicate that plateau cell number on MVG with these additions is substantially higher (5–30-fold) than plateau number on MV with the same additions. These are the only compounds so far found that will 'prime' wild-type cells. Addition of transamination inhibitors does not affect this growth. A possible explanation for these results is given in the Discussion.

Isolation of mitochondrial proteins

We investigated the possibility that the ability of *glg* and *msg* mutants to grow on MVG is due to an alteration in one or more of the mitochondrial proteins involved in transport. Mitochondrial proteins purified from a parental ditype segregant of X2180-1A and *glg4* were examined by one-dimensional SDS-acrylamide electrophoresis, and no differences could be seen between them. Such negative evidence does not, of course, rule out the possibility of an altered transport function.

Glutamate uptake by mutant mitochondria

The results of a glutamate uptake experiment involving mitochondria isolated from wild type, *glg4* and *msg1* are presented in Table III. As a control, the mitochondria were exposed to ^{14}C-labelled sucrose, which penetrates the space between the inner and outer mitochondrial membranes, and tritiated water, which can also penetrate the mitochondrial matrix. All time points were performed in duplicate, and for clarity the data in the table are presented both as averaged numbers of counts and as ratios of the incorporation of the three compounds. Standard errors on the ratios were calculated by means of an approximation derived from the first term of a Taylor series expansion. If $\bar{x}$ and $\bar{y}$ are the averaged number of counts in the numerator and the denominator, then the variance of the ratio is approximately [15]:

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

TABLE III

UPTAKE OF [^{14}C]GLUTAMATE, [^{14}C]SUCROSE AND $^{3}H_2O$ BY X2180-1A (WILD TYPE), *glg4* AND *msg1* MITOCHONDRIA

The data are presented as averaged counts in the first part of the table, and as ratios ± S.E. of the ratio in the second part (see text for details).

	(a) Averaged counts					
	[^{14}C]Glutamate		[^{14}C]Sucrose		$^{3}H_2O$	
	30 s	60 s	30 s	60 s	30 s	60 s
X2180-1A	248	326	1740	1670	187	346
glg4	180	610	979	1790	128	262
msg1	562	680	1983	2414	231	431

	(b) Ratios of counts ± S.E.	
	30 s	60 s
$^{3}H_2O$/[^{14}C]sucrose		
X2180-1A	0.170 ± 0.011	0.207 ± 0.038
glg4	0.131 ± 0.032	0.146 ± 0.066
msg1	0.116 ± 0.012	0.179 ± 0.051
[^{14}C]Glutamate/$^{3}H_2$)		
X2180-1A	1.326 ± 0.336	0.942 ± 0.228
glg4	1.406 ± 0.609	2.328 ± 0.981
msg1	2.433 ± 0.159	1.570 ± 0.288
[^{14}C]Glutamate/[^{14}C]sucrose		
X2180-1A	0.143 ± 0.038	0.195 ± 0.048
glg4	0.184 ± 0.084	0.341 ± 0.133
msg1	0.283 ± 0.030	0.282 ± 0.100

and since the numbers of observations N in the numerator and the denominator are the same, the standard error of the ratio $\sigma_{\bar{R}} = \sigma_R/\sqrt{N}$.

All compounds were taken up to a greater extent after 60 s than after 30 s except for [^{14}C]sucrose, which quickly saturated the intermembrane space of the wild-type mitochondria. The ratio of tritiated water to [^{14}C]sucrose was not significantly different between the three preparations at either of the time points.

It is possible to make eight comparisons of the rate of glutamate uptake in the mutant and wild-type cells, four for each time point. In each case, the ratio of [^{14}C]glutamate to [^{14}C]sucrose, and the ratio of [^{14}C]glutamate to $^{3}H_2O$, was greater in the mutants than in the wild type. Although none of the individual comparisons is significant (t-test), the probability that all eight would be larger by chance is only 0.004. This exceeds the 0.01 level of significance, and permits the conclusion that the rate of glutamate uptake by the *glg4* and *msg1* mitochondria is greater than that of wild-type mitochondria.

Genetics of the glg and msg mutants

The original mutants were isolated in the strain which was *MATa ade1 ade8*. This was mated to X2180-1A, *MATα*. Only tetrads which showed a regular segregation of the two adenine mutants were scored. Three classes of segregation were seen. In the first, consisting of eleven separately isolated mutants, the parental and non-parental ditype tetrads were 2:2 fast growth to no growth, and 4:0 slow growth, respectively. The tetratype tetrads were 1 fast: 2 slow: 1 no growth. In one case (*glg1*) there was a significant excess of parental ditypes, indicating linkage of the two genes. In no cases could linkage with a centromere (using the *ade1* gene) be detected. In these mutants two genes were present, each of which permitted slow

growth on MVG; both together permitted more rapid growth.

The second class of segregation involved an interaction between two genes. Three mutants, two *glg* and one *msg*, fell into this category. The parental ditype was again two fast to two no growth, but the non-parental ditype was 2 slow: 2 no growth, the tetratype was 1 fast: 1 slow: 2 no growth. The simplest interpretation of these results was that one gene permitted slow growth on MVG, and a second permitted fast growth but only in the presence of the first. In its absence the second gene did not permit growth.

In the remaining five of the mutants examined, the segregations were too complex to be analyzed, suggesting the presence of three or more mutant genes.

Because of the complexity of the segregation, tests for allelism gave equivocal results. Segregants of eleven *glg* mutants showing the first class of segregation carrying *ade1 MATa* and *ade8 MATα* were mated by cross-stamping from complete to minimal medium. The resulting diploids were checked for growth on MVG. The pattern obtained did not permit unequivocal classification into allelic groups. Surprisingly, *glg10* and *glg12* did not grow as diploids on MVG, although all segregants obtained when these diploids were sporulated grew well on MVG.

The segregation of *msg* mutants proved to be fully as complex as that of the *glg* mutants. Our preliminary conclusion is that a number of recessive mutant loci, some with epistatic effects, govern the ability to grow on MVG.

Discussion

The growth patterns seen in Fig. 4 and the inhibitor experiments lead to the following conclusions:

1. Transaminase inhibitors affect the malate-aspartate shuttle and gluconeogenesis pathway b in vivo. There are two pathways involving transaminations that become important when yeast is grown on a non-fermentable substrate. The first is gluconeogenic pathway B, in which the NADH generated by catabolism of the substrate in the cytoplasm in oxidized in the course of gluconeogenesis. This is not a shuttle. Only one equivalent of NADH is oxidized per equivalent of glucose produced. The second is the malate-aspartate shuttle, which will be expected to operate if an excess of NADH is produced in the cytoplasm. Both these pathways depend on active glutamate-oxaloacetate transaminase, and of the two transaminases the cytoplasmic one should be the more easily inhibited.

The growth of both wild-type and mutant yeast is consistent with the conclusion that there is an absolute dependence on transaminations when the cells are grown on non-fermentable substrates that generate cytoplasmic NADH. Both aminooxyacetate and cycloserine prevent growth on ethanol and lactate, and both prevent growth of the mutant strains on glycerol. These two transaminase inhibitors act to prevent the transfer of reducing equivalents into the mitochondrion in vivo. On MVD, however, where glycolysis is generating energy and the glycerophosphate-DHAP shuttle is transferring reducing equivalents into the mitochondrion, there is no measurable effect of the inhibitors. Most importantly, there is no effect of the inhibitors on acetate growth. Metabolism of acetate in the cytoplasm does not result directly in the production of NADH. There is therefore no necessity for the malate-aspartate shuttle to operate. Finally, there is no differential effect on the various media of the inhibitors *n*-butyl malonate, atractyloside or *N*-ethylmaleimide, which would be expected to inhibit other transport systems.

2. Hydroxycitrate inhibits the malate-citrate shuttle in vivo. The two inhibitors, aminooxyacetate and hydroxycitrate, together depressed growth on acetate. Cells grown on acetate will be expected to obtain their energy primarily through the synthesis of acetyl-CoA in the cytoplasm and its subsequent transfer into the mitochondrion through the acylcarnitine shuttle. The acetyl-CoA will generate citrate at the entry point of the Krebs cycle. Excess citrate, in turn, will be exported to the cytoplasm by the citrate-malate anion transport system. Oxaloacetate resulting from cleavage of the citrate will enter the gluconeogenesis pathway. Thus, if other pathways permitting malate to cross the mitochondrial membrane are inhibited, growth of cells on acetate should depend on the citrate-malate transporter. But if the malate-aspartate shuttle is permitted to operate, oxaloacetate can be gener-

ated directly from aspartate. Repression of growth on acetate is only expected in the presence of both inhibitors, which is what was observed.

3. n-Butyl malonate has an effect on malate-phosphate exchange in vivo. We noted an inhibitory effect on growth of *n*-butyl malonate on ethanol but not on pyruvate in the presence of aminooxyacetate. If the two inhibitors operating together shut down both the gluconeogenesis pathways shown in Fig. 1b, repression of growth would be expected on both substrates. We have no explanation for why growth on pyruvate is unaffected. But growth on acetate was unaffected by the presence of both inhibitors, so it is possible to conclude tentatively that *n*-butyl malonate has an in vivo effect on malate-phosphate exchange.

4. Shuttles involving transaminases can be circumvented by the addition of certain metabolic intermediates. Growth of wild-type cells on MVG is seen if low levels of pyruvate, oxaloacetate or α-ketoglutarate are added in either the presence or the absence of transaminase inhibitors. This can be explained if we note that these compounds can circumvent the transaminase-dependent shuttles. Addition of pyruvate should permit gluconeogenesis through the first gluconeogenesis pathway in Fig. 1b and perhaps through the malate-citrate shuttle, generating NAD^+ in the process. Addition of oxaloacetate should permit gluconeogenesis to operate directly. In both cases the NAD^+ generated will permit further metabolism of glycerol, so that some of the glycerol in the medium can be used for further growth. Finally, α-ketoglutarate can exchange with malate across the mitochondrial membrane. The malate can then drive the malate-citrate shuttle, generating both oxaloacetate for gluconeogenesis and NAD^+ in the cytoplasm. None of these pathways involve transaminations, and the prediction that none are inhibited by aminooxyacetate or cycloserine is borne out by the data.

5. The glg and msg mutants may have an altered malate-aspartate shuttle. These mutants do not show an enhanced rate of glycerol uptake, but they do show an enhanced rate of glycerol utilization, particularly when glutamate is present. Glycerol utilization is abolished by transaminase inhibitors, but not by inhibitors of other mitochondrial transport mechanisms. Growth of both mutants and wild type on ethanol and lactate is also prevented by transaminase inhibitors, while acetate growth is unaffected. There are two general possibilities for the increase in glycerol metabolism of the mutants. The first is that the proteins in the mitochondrial membrane responsible for glutamate-aspartate or malate-α-ketoglutarate translocation have been altered. No visible change in the mitochondrial proteins was seen, yet there was a significant increase in the rate of glutamate uptake by mitochondria in the two mutants tested. An alternative possibility for some of the mutants is that there may be metabolic blocks that increase the concentration of metabolites involved in the malate-aspartate shuttle. Both possibilities are being investigated further.

The preliminary results presented here are being used to design selection procedures for mutants in which shuttles are blocked, in order to investigate their effects on such fundamental cellular processes as glycolysis and gluconeogenesis. The value of obtaining such mutants has been pointed out by others [16].

Acknowledgments

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