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City University of New York PH.D. 1980

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by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1979

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This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

GLUCOSE REPRESSION RESISTANT MUTATIONS OF MITOCHONDRIAL BIOGENESIS IN YEAST

by

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Glucose repression resistant mutants have been isolated that are capable of growth on lactate in the presence of repressing levels of glucosamine. The mutants GSA^r-4 and GSA^r-5 are found to be resistant to glucose repression for cytochrome c oxidase and NADH: cytochrome c reductase. The other electron transport chain enzymes such as succinic dehydrogenase and succinic: cytochrome c reductase are still repressed by glucose to the same extent in the glucosamine resistant mutants as in the parent. The enzyme citrate synthase of the citric acid cycle is also repressed in the glucosamine resistant mutants. The enzymes maltase, galactokinase and α-galactosidase are also repressed by glucose to the same level in the mutants as in the parent.

The relationship between the rate-limiting enzyme of heme biosynthesis, δ-aminolevulinate dehydratase, and the derepression of the cytochromes has been investigated in GSA^r-4 and GSA^r-5. It has been found that while the mutants exhibit increased levels of glucose insensitive synthesis of

the cytochromes relative to the parent on 5% glucose, the level of δ-aminolevulinate dehydratase remains comparable to that in the parent. It is proposed that heme synthesis may play an indirect role in derepression of the cytochromes.

A detailed genetic investigation was conducted on the GSA^r-4 and GSA^r-5 mutants to identify the loci involved in the derepression process. Three unlinked nuclear loci, GRC1, GRC2, and GRC3 were found to be involved. Mutations at the GRC1 and GRC2 loci in GSA^r-4 and at the GRC1 and GRC3 loci in GSA^r-5 allow for the glucose insensitive synthesis of cytochrome c oxidase and NADH: cytochrome c reductase. The effect of each mutation on the respiratory chain was studied and no correlation was found between the presence of a specific grc mutation and the derepression of a specific segment of the respiratory chain. The presence of both grc1 grc2 in GSA^r-4 and of grc1 grc3 in GSA^r-5 was necessary for the glucose insensitive phenotypes.

The independent genetic control of the various complexes of the respiratory chain is interesting in light of the studies of the derepression of these enzymes. The two grc mutations we have isolated in GSA^r-4 and GSA^r-5 may be regulating the NADH: cytochrome c reductase and cytochrome c oxidase separately but since the effect of each gene is thought to be tightly linked to the other gene it may not be possible to study their expression in an environment devoid of the other gene. Also, our studies indicate that NADH:

cytochrome c reductase and succinic: cytochrome c reductase are regulated independently and only the gene(s) regulating NADH: cytochrome c reductase has been identified.

The role of the grc mutations in regulating mitochondrial biogenesis has been discussed. This regulation can occur on many levels from transcription of the structural gene to the insertion of the final polypeptide into the mitochondrial membrane. The regulation may also occur at the level of insertion of prosethetic groups. Several of these possible mechanisms have been discussed.

To Shiva Kant

Acknowledgements

I would like to express my thanks:

to Dr. D. Mowshowitz for providing the yeast strains used in this work.

to Dr. R. Needleman for providing me with his time in learning the enzyme assays and for many helpful discussions at the various stages of this work.

to Miss Susan Haupert for the excellent technical help during different stages of experimentation.

to Mr. Surinder Punj for typing this manuscript.

and deep appreciation to Dr. Corinne A. Michels for guiding this project to completion, giving me her kind help and patience during the years of my study at the City University of New York, and above all, for providing me with sincere appreciation of scientific knowledge.

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INTRODUCTION

I. INTRODUCTION

Since as early as 1919, it has been known that glucose, as well as a number of other sugars, inhibited the development of various fermentative capacities in bacterial and in yeast cultures (1). These early studies pointed out that inhibition produced by glucose was the most severe inhibition and the phenomenon therefore came to be known as the 'glucose effect'. The terms 'carbon catabolite repression' and 'catabolite repression' have also been used to describe this phenomenon. These terms were first used by Magasanik (2) to describe a similar phenomenon of repression in E.coli, and refers to the inhibition of certain degradative enzymes of carbohydrate metabolism by unknown product(s) or catabolites of sugar catabolism. In yeasts, glucose repression is a complex and multifaceted phenomenon in which a number of enzymes are regulated by the level of glucose in the growth media. In the following sections some of these glucose regulated functions and the possible mechanisms underlying their regulation are described.

II. MITOCHONDRIAL ASSEMBLY AND THE EFFECT OF GLUCOSE ON MITOCHONDRIA

The mitochondria are complex sub-cellular organelles with a classical double membrane structure. The outer membrane is smooth and somewhat elastic while the inner membrane has inward folds, or invaginations, called cristae. There is also a central cavity within the mitochondrion, the matrix, in which the Krebs cycle enzymes are located. In addition, the matrix

contains DNA molecules, mitochondrial ribosomes and the mitochondrial protein synthesizing machinery which acts independent of the cytoplasmic protein synthesizing system.

The inner membrane of the mitochondrion contains the enzymes associated with the electron transport chain and oxidative phosphorylation. The electron transport chain is organized into four distinct lipid protein complexes. The protein composition of each complex is different and on isolation of each complex *in vitro*, each catalyzes a specific segment of the overall oxidation reaction. The arrangement of various complexes as proposed by Hatefi et. al (3) is shown in Fig. 1(pg.32).

Complex I (NADH: coenzyme Q reductase) catalyzes the reduction of coenzyme Q by NADH. It contains covalently bound flavin mono nucleotide (FMN), and in most organisms four non-heme iron centers (4). However, yeast *Saccharomyces* contains less than four non-heme iron centers (5). Also, NADPH is oxidized directly without the intervention of NAD and the transhydrogenation reaction (See Fig. 1 pg. 32).

Complex II (Succinate: coenzyme Q reductase) contains succinic dehydrogenase, flavin adenine dinucleotide (FAD), a *b* type cytochrome (with absorption maximum at 557.5 nm, like that of cytochrome *b* in complex III) and certain non-heme iron centers.

Complex III (coenzyme QH_2: cytochrome c reducatse) catalyzes the reduction of cytochrome *c* by reduced coenzyme Q. The complex contains cytochromes *b*, c_1, and *c*.

Complex IV (cytochrome oxidase) catalyzes the oxidation of reduced cytochrome *c* by molecular oxygen. It is strongly

inhibited by cyanide, carbon monoxide and azide. The enzyme contains cytochromes a and a_3.

In addition to this, there is complex V, mitochondrial ATPase. This is an energy conserving complex and the energy released during electron transport chain is coupled to ATP synthesis (oxidative phosphorylation) by this complex. In yeasts, there are two sites where oxidative phosphorylation occurs: site II (between cytochrome b and cytochrome c) and site III (between cytochrome c and oxygen).

Most of the proteins of the citric acid cycle and the electron transport chain are coded for by nuclear genes as revealed by the presence of these proteins in chloramphenicol (mitochondrial translation inhibitor) grown cells and in neutral cytoplasmic petites (6). However, mitochondrial DNA does seem to code for all mitochondrial tRNA's, rRNA's and a limited number of protein subunits of some of the respiratory enzymes (6, 7, 8). The genetic and biochemical investigations of mit^- markers and antibiotic resistant markers indicate that the mitochondrial DNA is involved in the synthesis of cytochrome oxidase, cytochrome b and mitochondrial ATPase. (9-18). The most direct evidence that mitochondrial DNA codes for the mitochondrially synthesized polypeptides comes from the in vitro transcription and translation of isolated mitochondrial DNA to produce polypeptides which are precipitable using antiserum against mitochondrial membrane (19). Further, using antibodies directed specifically against cytochrome oxidase, Padmanaban et. al (20) have precipitated proteins synthesized in vitro

from a mitochondrial poly-A RNA fraction which was capable of specific hybridization to mitochondrial DNA.

The study of synthesis of those proteins that are coded for partly by nuclear and partly by mitochondrial genes has resulted in a great deal of information about the assembly and development of mitochondrial membrane. Also, it reveals how two different translation systems in the cell communicate with each other to synthesize a functional enzyme. These proteins will be briefly described here:

Cytochrome oxidase : Cytochrome c oxidase (from bakers' yeast and Neurospora crassa) is an oligomeric enzyme, consisting of seven polypeptide chains (21,22,23). The biosynthesis and assembly of these subunits results from the coordinated functioning of the cytoplasmic and mitochondrial protein synthesis (23,24,25). Three subunits (I-III) are synthesized on mitochondrial ribosomes whereas the remaining four subunits (IV-VII) are synthesized on cytoplasmic ribosomes (6). Recent evidence indicates that the mitochondrially made subunits I, II and III are coded for by OXI 3, OXI 1 and OXI 2 regions respectively of the mitochondrial DNA (26).

The mitochondrially made subunits are found to be relatively hydrophobic whereas the cytoplasmically made subunits are relatively hydrophilic (27). This could explain the localization of two largest mitochondrially made subunits in the interior of the enzyme complex and perhaps buried in the lipoprotein complex of the inner mitochondrial membrane (28).

Mitochondrial ATPase: The yeast enzyme has been extensively studied by Tzagoloff and co-workers. It is believed to contain at least ten distinct polypeptides (29, 30). Five of these subunits, associated with the F_1 part of the mitochondrial ATPase complex, are synthesized on cytoplasmic ribosomes and are present in neutral cytoplasmic petites (6, 31, 32). The oligomycin sensitivity conferring protein is also a cytoplasmic product (33). The four hydrophobic subunits in contrast are made in the mitochondria (34, 35). These are coded for by pho-1, pho-2, oli-3 (for 6a and 6b) and oli-1 (for 9) regions on mitochondrial DNA (26).

Cytochrome b: The apo-cytochrome b (the protein part of the cytochrome without the heme) is coded for by cob-box region on mitochondrial DNA (for review see 26). Mutants lacking a functional cytochrome b map in seven discrete clusters within the cob-box region of mitochondrial DNA. These seven clusters are non-adjacent to each other and are referred to as box 1-7. Of these, mutations at box 4/5, 1 and 6 fall into a single complementation group while 2,3 and 7 fall into a different complementation group. The coding sequence for cytochrome b is believed to be put together by splicing and linking together4,5,1 and 6 regions in a specific manner. This situation is similar to many eukaryotic systems where coding sequences are often interspersed with non-coding sequences (36, 37). Regions 2, 3 and 7 on the other hand act independently in the regulation of cytochrome b and cytochrome oxidase.

a. Glucose effect on mitochondrial structure

The effect of glucose on mitochondrial structure and biogenesis have been studied in detail by numerous workers. One of the big advantages of this system is that cells grown in glucose satisfy their energy requirements by glycolysis and not by respiration and hence, mitochondrial function, at least as far as respiration and energy transduction are concerned, becomes dispensable. Once glucose is exhausted from the medium, the cells elaborate functional mitochondria. Thus, by following glucose repression and derepression of the mitochondrial structure, one can gain insight into the elaboration of this complex organelle.

It has been found that the mitochondria of glucose grown cells are fewer in number and exhibit very little inner membrane organization (38-41). Also, mitochondria in repressed cells appear considerably larger, more irregular in shape than the relatively symmetrical ones seen in cells released from glucose repression or in cells growing exponentially on ethanol or lactate as a carbon source (42). Electron microscope studies of yeast grown in 5% glucose show that it is not until the transition from the exponential phase to stationary phase that large numbers of mitochondria appear. The cristae of these mitochondria are well organized and exhibit a normal function. Glucose is found to be more effective in producing repression than any other fermentable carbon source.

The effect of glucose on mitochondrial structure and enzymes is more severe than on the total oxygen uptake of whole cells (43). There is a sharp decrease in the oxygen uptake on

transferring the cells from lactate to glucose. This decrease is faster than can be explained by the absence of oxidative ability in newly arising cells, since maximum decrease occurs in absence of growth. This decrease is found to be preceded by a sharp decrease in the levels of mitochondrial oxidases, dehydrogenases and cytochromes. Adaptation to growth on ethanol produces an increase in the level of oxygen uptake preceded by an increase in the level of these enzymes. Hence, it seems that during the presence of glucose in the medium, existing mitochondrial machinery is dismantled and upon removal of glucose perhaps reassembled and reorganized. Schatz and his co-workers (44) have postulated that respiratory adaptation involves conversion of mitochondria like particles in anaerobically glucose grown cells into functional mitochondria. In this regard, they have named the anaerobic particles 'promitochondria', analogous to 'proplastid', chloroplast precursors of light deprived plants (45). In support of this view, Schatz et. al showed that when promitochondria were specifically labelled with ^{3}H-leucine in the presence of cycloheximide (cytoplasmic translation inhibitor) and allowed to adapt in unlabelled medium after the removal of the antibiotic, the label was associated with cytochrome oxidase stain, as judged by electron microscopic autoradiography (46). Thus, respiratory adaptation, according to these authors, does not involve proliferation of new mitochondria but structural differentiation of pre-existent mitochondria. Glucose repression may involve a similar differentiation pattern.

b. Glucose effect on mitochondrial enzymes

The changes in mitochondrial structure correlate with the change in the activity of mitochondrial enzymes (44, 47, 48, 49). All enzymes are subjected to varying degrees of repression by glucose and undergo derepression upon removal of glucose from the medium. In addition, effect of glucose has also been looked at for some cytosolic enzymes because they are either intimate part of carbon metabolism (gluconeogenic enzymes) or because they are important in cytochrome synthesis (heme synthesis enzymes).

The Krebs cycle functions both in the catabolic generation of ATP and in anabolic synthesis of important biosynthetic precursors. The major biosynthetic products are derived from α-ketoglutarate (which serves as the source of glutamate) and oxaloacetate (this provides aspartate). These by-products in turn give rise to various amino acids. The other essential product of the Krebs cycle is succinyl-coA, a precursor of both methionine and porphyrins. Such diversity of functions has produced a complex set of repressive controls for the synthesis of the enzymes involved.

The change in the activity of the Krebs cycle enzymes and the gluconeogenic enzymes upon aerobic growth on several different carbon sources has been studied by Polakis and Bartly (47). It has been found that under high glucose growth conditions, with low activity of the electron transport chain enzymes and no well-developed mitochondria, a low but significant activity of the Krebs cycle enzymes is observed. Under fully

derepressed conditions of growth on ethanol and acetate, an increase in the activity of these enzymes is observed but the derepression is not as dramatic as for the glucose regulated enzymes of E. coli or even other yeast enzymes. For instance, citrate synthase shows a derepressed value of 300-400 % of the repressed level while aconitase shows only a 20% increase in the activity on transfer to ethanol.

The repression observed for the enzymes of gluconeogenesis is more complete (47, 50). It is observed that isocitrate lyase and malate synthase are repressed to 1/150 and 1/100 that of non repressed cells. Both of these enzymes function only in gluconeogenesis. However, enzymes like malate dehydrogenase and alcohol dehydrogenase, which function both in gluconeogenesis from ethanol and in the Krebs cycle exhibit a different set of controls. Both enzymes are present as isozymes which exhibit differential sensitivities to glucose. The enzyme malate dehydrogenase is found to exist in two different forms in the yeast cell and both forms respond differently to external glucose conditions (51). The mitochondrial malate dehydrogenase resides in the mitochondrion and participates in the Krebs cycle. It is repressed by only high concentrations of glucose. The cytoplasmic malate dehydrogenase functions in the gluconeogenic pathway and is affected by even slight amounts of glucose and is also inactivated by glucose (52). Similarly, alcohol dehydrogenase is present as three isozymes in the yeast cell. ADH1 is cytosolic and constitutive, ADHII is cytosolic and glucose repressible, and ADM is mitochondrial (53,54).

The enzymes of the respiratory chain, including the mitochondrial ATPase, are subject to repression by glucose (43,55). The repression varies from strain to strain and from one complex to another. For instance, the derepression for NADH: cytochrome c reductase is to 200-300% of the repressed level while for some cytochromes it is about 1000%.

Of the various cytochromes subject to glucose repression, only kinetics of glucose repression of cytochrome c synthesis has been looked at in great detail (56, 57). Cytochrome c is a mitochondrial protein coded for by a nuclear CYC1 gene (58). In Saccharomyces cerevisiae addition of 5 or 10% glucose to a derepressed culture leads to a decrease in the rate of synthesis of cytochrome c with a half life of 2 min. (56). The level of CYC1 mRNA was followed using hybridization to cloned CYC1 DNA. The rate of transcription of the CYC1 gene reduces to the repressed levels for this enzyme within a 2-5 min period following addition of glucose (57).

The kinetics of formation of different respiratory enzymes and cytochromes during release from glucose repression have been studied by Kim and Beattie (55) and Perlman and Mahler (72). It has been found that various enzyme complexes of the mitochondrial membrane are assembled in the membrane in an asynchronous way (55). Each enzyme starts to increase at a unique time in the growth curve, increases at a characteristic rate and reaches a maximum at a unique time. Proteins of both cytoplasmic as well as mitochondrial origin are synthesized during this derepression process and even the synthesis of

different components of the same enzyme seems to be asynchronous. For example, synthesis of cytochrome oxidase involves first the formation of cytoplasmically made subunits followed by synthesis of the mitochondrially made subunits of this enzyme. Furthermore, these subunits may accumulate to a limited extent in the cell before their integration into a functional enzyme complex. As for the sequence of elaboration of different enzymes, mitochondrial ATPase is elaborated first, followed by other enzymes of the respiratory chain with cytochrome oxidase being the last enzyme to be induced.

The derepression process is believed to take place in two distinct phases (42):

1) Fermentative phase: is characterized by the synthesis and accumulation of various respiratory enzymes (except for cytochrome oxidase). This phase begins even when the external glucose concentration is as high as 0.4%. The products of mitochondrial translation system and components of the respiratory chain are not required for this phase as illustrated by its insensitivity to Antimycin A.
2) Oxidative phase: takes place after respiration has begun and this phase depends upon the mitochondrial respiratory function. This phase is sensitive to chloramphenicol and is found not to occur in cytoplasmic petites. Only cytochrome c oxidase and oxygen uptake appear to undergo derepression during this phase.

c. Glucose effect on heme synthesis

Cytochromes are heme containing proteins and since cytochromes are subject to repression by glucose, it is conceivable that repression may be occuring at the level of heme synthesis. Two of the enzymes involved in the synthesis of heme are α-aminolevulinate synthetase (alv synthetase) and δ-aminolevulinate dehydratase (alv dehydratase). These two enzymes catalyze the first two steps in heme synthesis and both are found to be glucose repressed (59, 60).

Alv synthetase is an exclusively mitochondrial enzyme. The derepression of this enzyme from glucose is abolished in the presence of cycloheximide and chloramphenicol.

Alv dehydratase is a cytosolic enzyme and believed to be the rate limiting enzyme in heme synthesis (61). This enzyme is present in lower levels than Alv synthetase. Its release from repression is rapid. The derepression is inhibited by cycloheximide (inhibitor of nuclear transcription in yeast) but not by chloramphenicol. It has been found that enzyme formation in response to derepression requires the participation of both nuclear transcription and cytosolic translation (61).

d. Glucose effect on mitochondrial DNA

Another apparent consequence of glucose effect is the decrease in the mitochondrial DNA content of the cells (40,41,62). It is proposed that there are fewer mitochondrial genes in repressed cells (62) and the transmission of these loci to the daughter progeny is markedly reduced from the glucose grown parent (63, 64).

III GLUCOSE EFFECT ON ENZYMES OF CARBOHYDRATE METABOLISM

The synthesis of various enzymes of carbohydrate metabolism is affected by glucose. These enzymes include the galactose, maltose, sucrose and melibiose fermenting enzymes. All are relatively clearly genetically defined and lend themselves easily to biochemical investigations.

a. Galactose fermentation

The genetics of galactose fermentation is best understood. Four enzymes are found to be required for galactose utilization, of which one is a transport protein. The other three enzymes, galactokinase, transferase and epimerase are coded for by GAL1, GAL7 and GAL 10 respectively (65) and together constitute the gal region. All the three enzymes are co-ordinately induced in the presence of galactose (66) and the three gene loci map in a tightly linked cluster (67). There are at least three loci that are believed to regulate the expression of gal region: GAL 80(i), GAL 81 (4 and c) and GAL3. The galactose induced enzymes seem to be synthesized constitutively in gal80 mutants. Also, the GAL81 gene product appears to be a constitutively synthesized protein (68,69). Fine structure mapping of this region indicates the presence of only one structural gene (70) where two loci (GAL4 and GALc) were thought to be present. The following sequence of events has been proposed in the regulation of galactose fermentation (68,71,72,73). The GAL80 gene is believed to code for a repressor protein while GAL81 gene codes for another protein that regulates the expression of the galactose fermentation structural genes positively. In absence of galactose, the GAL80 gene product combines with GAL81 gene

product turning off the synthesis of the GAL 1, 7 and 10 genes. However, the presence of galactose removes the repressor from the medium leaving the GAL81 gene product free to turn on the synthesis of the structural genes.

Glucose represses the synthesis of the first enzyme in galactose utilization, galactokinase, in a manner similar to that of β-galactosidase repression in E.coli (74). Glucose affects the synthesis of galactokinase in three ways. First, an inducer exclusion effect, wherein glucose prevents the uptake of galctose (inducer) into uninduced cells; this can be overcome by inducing the cells prior to glucose addition or by using large quantities of the inducer. Secondly, addition of glucose to cells growing on galactose results in a severe but temporary inhibition of galctokinase synthesis. This phase is called transient repression and is followed by resumption of enzyme synthesis at a somewhat reduced rate called catabolite repression. In sharp contrast to E.coli, if the yeast cells are grown on glucose and then transferred to galactose medium, 3-5 hr time lag is observed before the induction of galactokinase activity as compared to 6-8 min time lag if the cells are previously grown on lactose. In E. coli, no such dependence on the prior carbon source is seen. The 3-5 hr time lag for enzyme synthesis in yeast probably results from the repressed state of the mitochondrial apparatus.

In addition to catabolite repression, glucose also causes catabolite inactivation of the galactose uptake system (75). This inactivation is thought to occur by a decrease in

the affinity for galactose of the galactose uptake system. The inactivation can be prevented by addition of cycloheximide, indicating the participation of a protease either activated by glucose or whose synthesis is induced by glucose.

b. Maltose fermentation

The utilization of the sugar maltose by yeast cells requires the presence of maltase and maltose permease (76,77). Maltose fermentation requires the presence of any one of the seven polymeric genes (MAL1 to MAL7) (78,79,80). MAL2, MAL4 and MAL6 (and probably all the other MAL genes) are the regulatory genes for maltase (81-85) and the structural gene for maltase has not been identified as yet. ten Berge et.al (81,82) have isolated different classes of mutants for the MAL6 gene and found that although some of the mutants produce very low levels of maltase and some others produce maltase constitutively, the maltase produced is not biochemically different from wild type maltase. Had MAL6 been the structural gene for maltase, any mutation in this gene would give a structurally altered form of maltase. Similar results were also reported for MAL4 gene (84). Some recent reports indicate that each MAL gene is linked to the structural gene of maltase but further work is needed to clarify this situation (86).

Presence of maltose in the medium induces the production of maltase and maltose permease while glucose represses their synthesis. Glucose also causes catabolite inactivation of maltose permease (76). In media containing both glucose and maltose, release from repression occurs upon consumption of

glucose. Adaptation to growth on maltose or galactose (as the case may be) is found to be inhibited in the presence of respiratory inhibitors such as erythromycin and during anaerobiosis (87,88).

c. Other fermentable sugars and catalase

Invertase, another enzyme of carbohydrate metabolism, hydrolyses sucrose into fructose and glucose. The presence of any one of a series of six non-allelic SUC genes is necessary for the fermentation of sucrose (89). However, there is no clear evidence to prove whether these genes are structural or regulatory in function. A single point mutation in one of the genes, allelic to SUC3 gene, leads to loss of both external and internal (see below) invertase. This suggests either a regulatory role for this gene or that it codes for a polypeptide common to both the enzymes (90).

Yeasts contain two classes of invertase, an internal and an external (cell bound but not extracellular). In the presence of glucose, only internal invertase is found (91). Under derepressed conditions the external type dominates (91,92). It is suggested that these two kinds are immunogically related and at least one subunit is common to both of these multimeric enzymes (93).

The fermentation of sugar melibiose requires the participation of the enzyme α-galactosidase. A series of five polymeric MEL genes are believed to be involved in the fermentation of this sugar (94). There is no clear evidence whether these MEL genes are structural or regulatory in

function. The enzyme α-galactosidase is repressed by glucose but inducible by both melibiose and galactose. It is under the regulation of GAL structural genes (i.e. GAL80, GAL81 and GAL3) (95).

The enzyme catalase is a heme containing enzyme and therefore the study of this enzyme may reveal how other related heme containing enzymes (such as cytochromes) are regulated. There are two types of catalase in the yeast cell, catalase A and catalase T. Both are sensitive to glucose repression but exhibit differential sensitivies to glucose (96,97). Catalase A is more severely repressed and requires respiratory competence for its derepression from glucose. Catalase T is less sensitive to repression by glucose. The results indicate that catalase A and catalase T are under the control of different regulatory systems.

IV REGULATION OF GLUCOSE REPRESSION

The effect of glucose on various aspects of cell metabolism and mitochondrial structure has been discussed in the preceding sections. Although a large class of mutants with altered sensitivities to glucose have been isolated, none of them reveal a clear picture of the mechanism of glucose repression. In the following studies an attempt has been made to group together the facts that are known at present concerning the regulation of this phenomenon. Although glucose also causes catabolite inactivation of certain enzymes, this topic is restricted to the mechanism of glucose repression only.

A great deal of knowledge regarding the mechanism of catabolite repression comes from the study of the lac operon in

E. coli. The three enzymes of the lac operon are coordinately induced in the presence of lactose and glucose inhibits their synthesis. However, the repression of β-galactosidase (product of lac z gene of the lac operon) has been found to be overcome by addition of extracellular cyclic adenosine 3', 5'-mono phosphate (cyclic AMP) (98). Also, there is a direct correlation between the level of cyclic AMP and the degree of repression of this enzyme (99). The lower the level of cyclic AMP, greater the repression of this enzyme. Another protein factor seems to be involved in the regulation of β-galactosidase, catabolite gene activator protein or CGAP or CAP (100). The action of cyclic AMP and CGAP on the lac operon in E.coli can be summarized as follows: lac promotor contains two distinct physical regions; one site binds to RNA polymerase and the other to CGAP-cyclic AMP complex. In the presence of lactose, CGAP-cyclic AMP complex binds to the lac promotor and in doing so enhances the ability of RNA polymerase to transcribe the lac operon more effectively (101). In the absence of cyclic AMP (as is the case in the presence of glucose), transcription of the lac operon occurs at a reduced rate.

a. Role of cyclic AMP in yeast

It has been shown that the level of cyclic AMP is 6-8 fold higher (102) or as much as 20 fold higher (103) in yeast cells grown on non glucose medium than in the cells grown on 5% glucose. A direct correlation between the intracellular levels of cyclic AMP and the activity of alcohol dehydrogenase has been shown (103). Cyclic AMP has also been

shown to reverse the repressive effects of glucose on maltase synthesis (104) and on mitochondrial respiration (105) in yeast protoplasts. This phenomenon is found to be sensitive to chloramphenicol (104). In higher concentrations (10 mM) cyclic AMP may also cause stabilization of at least one enzyme in vivo (106).

A wide variety of enzymes can be relieved from the effects of glucose repression by addition of extracellular cyclic AMP. These include mitochondrial enzymes ∝-aminolevulinate synthetase, malate dehydrogenase, NADH: cytochrome c reductase and cytochrome oxidase (106). The kinetics of derepression are indistinguishable from the cells transferred from glucose to ethanol. Other nucleotides like cyclic GMP, ADP, 5'-AMP or 5'-GMP are inactive in producing derepression.

A cyclic AMP binding protein has been isolated from some yeast strains which could have a role similar to the CAP protein in E. coli but no evidence is available (107). The level at which cyclic AMP may be regulated is also not known. Saccharomyces fragilis contains a membrane bound adenyl cyclase whose activity is 7-8 fold higher in lactate grown cells as compared to cells grown in glucose (108). However, S. cerevisiae and S. carlsbergensis could not be shown to have any such cyclase activity by Sy and Richter (108), but other workers have found a Mn-dependent adenyl cyclase in S.cerevisiae (109). A cyclic AMP phosphodiesterase may have a regulatory role in S. carlsbergensis (110).

b. Role of intermediary metabolites

The role of glycolytic intermediates in glucose repression has been studied by Ciriacy (111). A number of mutants in which glycolysis is blocked at a particular step have been isolated. Such mutants with increased amounts of various glycolytic intermediates (due to a defect in the activity of the enzyme that catalyzes their utilization) show normal repression for succinate dehydrogenase, isocitrate lyase and fructose-1,6-bisphosphatase. This reveals that glycolytic intermediates are not the control molecules in glucose repression.

Several glucose analogues such as 2-deoxyglucose and D-glucosamine produce repression similar to that exerted by glucose (112, 113). 2-deoxyglucose in yeast is phosphorylated and then converted to UDP-deoxyglucose (158). D-Glucosamine in yeast is only phosphorylated (159). This suggests that it is either glucose or a phosphorylated derivative of glucose that is the controlling molecule in glucose repression. Thus, the terms catabolite repression and carbon catabolite repression should be reserved only for bacteria and instead the term glucose repression should be used to describe this phenomenon in yeast.

c. Role of the mitochondrion

Since many of the enzymes subject to glucose repression reside and are functional in the mitochondria and since the mitochondrial DNA itself is subjected to

glucose repression, it is of interest to know whether the mitochondrion contributes to glucose repression. This regulation could be achieved in terms of either functionally or genetically competent mitochondria. In a study done by Haubmann and Zimmermann (114), normal, respiratory competent (RHO) strains were found to have lower specific activities for maltase and invertase as compared to their counterpart mutants (rho). It was postulated that some factor transcribed on mitochondrial DNA and then translated in the cytoplasm was exerting a negative influence on the activity of these enzymes. In certain other cases there may actually be an increase in the activity of some enzymes in respiratory proficient strains as compared to respiratory deficient strains (115, 116). The derepression of catalase A is believed to be dependent upon the respiratory competence and availability of ATP from the mitochondrion (97). However, mitochondrial function or an intact mitochondrial genome and the products of its expression are not required for the derepression of δ-aminolevulinate dehydratase (61).

The influence of the mitochondrion on some of the respiratory enzymes can occur either by nuclearly coded or by mitochondrially coded repressor proteins (117, 118). Nuclearly coded mitochondrial 'organizers' may exist. A number of nuclear mutations having the petite (pet) phenotype have been studied (117). These mutants retain mitochondrial protein synthesis but are pleiotropic in their effects upon the synthesis of cytochromes $\underline{c}_1$, $\underline{b}$, $\underline{aa}_3$ and mitochondrial ATPase. It is suggested that a nuclearly coded

mitochondrial protein is involved in mitochondrial assembly.

A mitochondrially made repressor is also thought to be involved in the regulation of mitochondrial proteins in fungi other than yeast. Evidence of this comes from the work of Barath and Kuntzel (118) who found that growth of Neurospora crassa cells in ethidium bromide and chloramphenicol leads to an increased production of mitochondrial enzymes. Also, Keyhani and Keyhani (119) have found that low concentrations of adriamycin (cytotoxic compound) in exponentially growing yeast Candida utilis induces cytochrome biosynthesis and also increases the incorporation of radioactive leucine in all classes of mitochondrial polypeptides. On the basis of this observation it has been suggested that during exponential growth of yeast cells, there is an increase of all classes of mitochondrial proteins, among which is also a repressor mitochondrial protein. This repressor protein, in turn, regulates the expression of other respiratory enzymes. However, if the cells are grown in the presence of mitochondrial inhibitors, the synthesis of this repressor protein is turned off thereby leading to a temporary increase in mitochondrial proteins.

V TRANSCRIPTIONAL, POST-TRANSCRIPTIONAL OR TRANSLATIONAL CONTROL

Glucose repression could be operating at the level of transcription or could exert its effect post-transcriptionally, translationally or post-translationally. As noted for β-galactosidase in E. coli, the regulation seems to occur at the level of transcription. The problem, however, is more complex

for eukaryotic organisms such as yeast in which mRNA is longer lived with an average half life of 20 min (120-126). Thus, the regulation in systems with longer lived mRNAs should be more effective if it operates at the translational level. On the other hand, Sripati and Warner (126) reported that a significant amount of yeast mRNAs have half life of 3-6 min., in which case transcriptional control could be equally effective. The evidence available presently indicates both sets of controls.

a. Transcriptional and post-transcriptional control

Cytochrome c in yeast is believed to be regulated both at the level of transcription and post-transcriptionally by glucose (56, 57). The addition of glucose to a derepressing culture results in an immediate decrease in the rate of synthesis of CYC1 mRNA. This decrease takes place with a half life of 2 min. Also, the amount of cytochrome c protein is repressed within the same time period. However, the half-life of CYC1 mRNA as measured by DNA-RNA hybridization is about 13 min (57). Clearly, this mRNA is not available for translation. It is proposed that, on addition of glucose, the rate of production of CYC1 mRNA is reduced and the existing mRNA for CYC1 is rendered untranslatable by an endonucleolytic cleavage. Other forms of post-transcriptional controls can also be available to the cells but must be operative in the presence of glucose. Other enzymes believed to be regulated at the level of transcription are α-aminolevulinate synthetase and δ-aminclevulinate dehydratase (61).

The enzyme maltase is regulated both at the level of transcription and translation in yeast. The effect of actinomycin D on the induction of maltase and its repression has been studied in protoplasts of Saccharomyces carlsbergensis (77). The induction of maltase synthesis is actinomycin D sensitive only during the first 90-120 min following inducer addition. This suggests that the mRNA coding for maltase is stable. However, addition of glucose during the actinomycin D insensitive phase also represses maltase synthesis and therefore glucose must be exerting its influence post-transcriptionally. High glucose concentrations during the actinomycin D sensitive phase also reduced the level of mRNA indicating its control on transcription.

b. Translational control

The enzyme invertase is thought to be regulated both at the level of translation as well as post-translationally. The effects of lomofungin, an RNA synthesis inhibitor, cycloheximide and fructose on the synthesis of invertase have been compared (127). Both cycloheximide and fructose caused an immediate inhibition of invertase synthesis. In contrast, lomofungin inhibited invertase synthesis after a 40 min delay, a response consistent with the inhibition of synthesis of a messenger of moderate half-life. The glucose repression of invertase synthesis therefore resembles the inhibition produced by a protein synthesis inhibitor indicating that, as for maltase, glucose repression may be occuring at the translational level. Until the level of SUC

mRNA can be measured by hybridization, other post-transcriptional controls can be advanced. A recent study gives evidence for the post-translational control of this enzyme. It was found that glucose prevented the addition of carbohydrate moiety to the polypeptide segment of invertase and thereby preventing the secretion of mature invertase (128).

c. Possible mechanisms of translational and Post-translational control in Mitochondrial Biogenesis

Since, even in presence of glucose, yeast cells are found to have mitochondria like particles, or promitochondria, it can be conceived that restoration of functional ability to these promitochondria on removal of glucose involves (i) changes in inner mitochondrial membranes and (ii) reorganization of the enzymes within the mitochondria. It is not known whether development of inner mitochondrial membrane is a prerequisite for the activity of membrane associated glucose sensitive enzymes or restoration of activity to these enzymes precedes the development of the inner mitochondrial membrane.

Translational and post-translational regulation of mitochondrial biogenesis could take place at several levels in the yeast cell: 1) by controlling heme synthesis and 2) during elaboration of different subunits of a multimeric respiratory enzyme. These controls will be briefly described here.

1. Heme synthesis: The possible regulation of mitochondrial biogenesis by heme was first put forward

by Sugimura (129) and in rat liver by Beattie (130). Beattie showed that administration of porphyrinogenic drugs to rat liver mitochondria increased the rate of synthesis of cytochromes. Since this effect was abolished in the presence of inhibitors of heme synthesis, it appeared as if the effect was due to elevated levels of cellular heme caused by the drugs. Cabral et. al (131) have shown that the rate of mitochondrial protein synthesis is markedly reduced in a mutant lacking ∝-aminolevulinate synthetase. Also, mutants lacking ∝-aminolevulinate synthetase exhibit a deficiency in the synthesis of all cytochromes. However, cytochrome c synthesis was restored in the mutants supplemented with ∝-aminolevulinic acid (132).

A role for heme synthesis in the regulation of cytochrome oxidase during respiratory adaptation was further suggested by Charlampous (133). Heme deficiency may also have effects at the integration level of certain cytochromes into the inner mitochondrial membrane. Ross and Schatz (134) have shown that the apo-protein of cytochrome c_1 may accumulate in the cytoplasm of the heme deficient mutants, as it does in the anaerobic cell. Also, in an earlier study (135) it has been reported that addition of heme a to isolated petite mitochondria was sufficient to reconstitute cytochrome oxidase activity. It is not clear whether this reconstitution of activity indeed reflected formation of cytochrome oxidase holoenzyme.

Interestingly, regulation of globin (of hemoglobin) synthesis in mammalian reticulocytes is controlled by the availability of hemin; in the absence of hemin, reticulocyte polyribosomes disaggregate as a result of a block in re-initiation of protein synthesis (for review see 136). In the absence of hemin, a repressor (called 'Hemin controlled repressor' or HCR) acts at the level of translation of protein synthesis or specifically on (met-$tRNA_f$.40S) ribosomal initiation complex and stops protein synthesis by inactivating an initiation protein eIF-2. Addition of hemin inactivates their repressor. It is possible that glucose repression of cytochromes occurs through regulation of heme in a similar manner.

2. Elaboration of different enzymes and different subunits of one enzyme:

The presence of subunits of cytoplasmic and mitochondrial origin in a number of mitochondrial enzymes suggests that some mechanism exists to insure their proportionate production in the cell. The first indication of this kind of regulation came from the work of Tzagoloff (137), who found that the rate of mitochondrial protein synthesis could be greatly increased if the cells were first preincubated in chloramphenicol, which allowed for the accumulation of proteins synthesized in the cytoplasm It was postulated that this accumulation of cytoplasmic products stimulated the production of their mitochondrial counterparts. Further, it was also postulated that proper

integration of cytoplasmically made proteins requires production of mitochondrially made subunits. Hence, a sort of inter-dependence of the two protein synthesizing systems occurs during mitochondrial biogenesis.

The regulation of the protein subunits of respiratory enzymes can again occur at the levels of transcription, post-transcriptionally and/or at the level of translation. In the previous section it was shown that cytochrome c synthesis is regulated transcriptionally as well as post-transcriptionally by glucose (56, 57). Even though the information about the regulation of other respiratory enzymes is limited, it is possible that they may be regulated in a similar manner.

The co-ordinated regulation of production of mitochondrial and cytoplasmically made subunits of respiratory enzymes can occur at the level of lipid synthesis of mitochondrial membranes. The respiratory adaptation in yeast cells is accompanied by an increase in the levels of long chain unsaturated fatty acids (ergosterol and ubiquinone) that constitute the lipid inner membrane of mitochondria (138, 139).

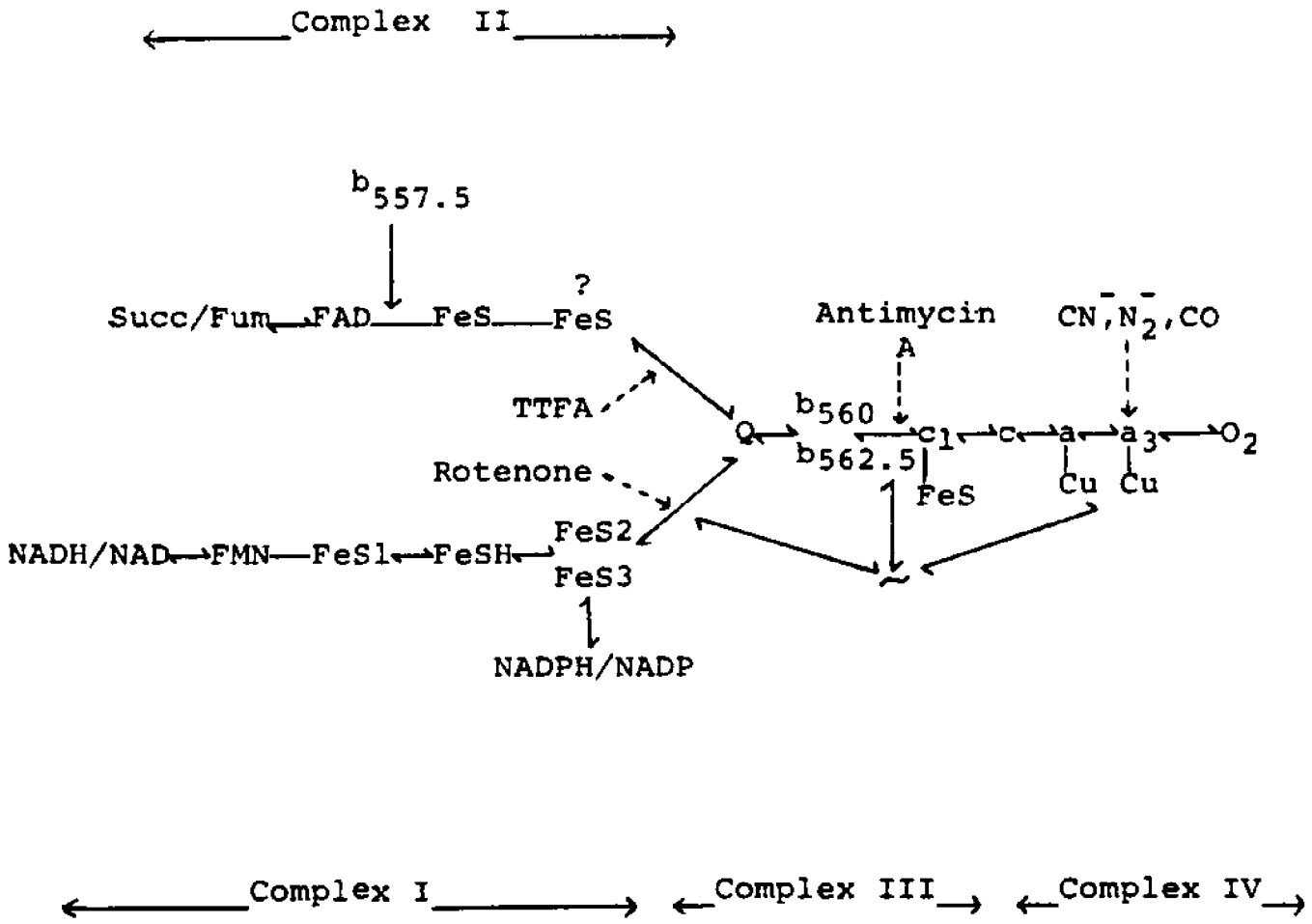
Another important feature of enzymes assembled from both the mitochondrial and cytoplasmic translation systems is the transport of their cytoplasmically made subunits into the mitochondria. Study of the uptake of subunits of mitochondrial ATPase show that precursors are synthesized on cytoplasmic ribosomes and their transport into the mitochondria is accompanied by their proteolytic cleavage into the mature subunit (164). Glucose repression of such

respiratory enzymes of dual origin can occur during the transport and/or maturation of their cytoplasmically made subunits.

Presuming such tight interdependence between the cytoplasmic and mitochondrial translation systems, it can be thought that repression of synthesis of either mitochondrially made subunits or cytoplasmically made subunits would lead to repression or shut down of the respiratory chain. This would mean utilization of fewer key effector molecules in repression.

The physical effects of glucose repression have been discussed in the preceding sections. However, relatively little is known about the genes involved in the phenomenon of glucose repression in yeasts. In order to understand the genetics, mutants must be isolated that are altered in the synthesis of enzymes in the presence of glucose. Although a large number of mutants have been isolated to this purpose none of them have clearly identified any gene locus (loci) that regulates glucose repression. The present study was undertaken to identify the gene loci involved in glucose repression of mitochondrial biogenesis and their possible participation in mitochondrial assembly.

Fig. 1. Mitochondrial electron transport system. Abbreviations: Succ, succinate; Fum, fumarate; FAD-Fes, iron-sulfur flavoprotein subunit of succinic dehydrogenase; Fe-S, iron sulfur protein; FeS1, FeS2, FeS3 and FeS4, respectively iron-sulfur centers 1,2,3 and 4; Q, Coenzyme Q; b, c_1, c, a, a_3, respectively cytochromes b, c_1, c, a, a_3; TTFA, thenoyl-trifluoroacetone. The various respiratory inhibitors are shown by broken lines. For details see text. (From Ref. 3.)


Complex II
b557.5
Succ/Fum
FAD
FeS
?
FeS
Antimycin
A
CN⁻, N₂⁻, CO
TTFA
Q
b560
b562.5
c1
c
a
a3
O2
FeS
Cu
Cu
Rotenone
NADH/NAD
FMN
FeS1
FeSH
FeS2
FeS3
NADPH/NADP
Complex I
Complex III
Complex IV

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Strains: The following haploid strains of yeast Saccharomyces carlsbergensis were used: M18-19F (α -ade1 trp5 MAL6- constitutive mgl GAL SUC) and M18-23E (a ade1 ura MAL6- constitutive mgl GAL SUC). Both were obtained from Dr. Deborah Mowshowitz, Columbia University, Department of Biological Sciences, New York, N.Y.

Media: YP, containing 1% Difco bacto-yeast extract and 2% Difco bacto-peptone was used as the basic media. To this the final concentrations of 2 or 5% glucose, 2% lactic acid and 0.1% D- glucosamine were added as appropriate for each experiment.

Isolation of mutants: The mutants were isolated using nitrous acid (HNO_2) as a mutagen in the following manner. The parent strain was grown to saturation in liquid YP + 2% glucose. The cells were harvested, resuspended in an equal volume of water and starved at $4^{\circ}C$ for one to four days. The starved cells were then harvested and concentrated 10 fold into water. Sodium nitrite was added to a final concentration of 6 μg/ml. The cells were mutagenized to give about 10-20% survival rate. Following mutagenesis, the cells were washed twice with buffer (potassium phosphate buffer, pH 6.8), resuspended in YP + 2% glucose medium and divided into a number of separate cultures. Each culture was allowed to grow to saturation at $30^{\circ}C$ and, from each, a heavy inoculum of

cells was plated on to plates containing YP + 2% lactate + 0.1% glucosamine. The plates are incubated at 30°C until colonies appeared. One colony from each culture was selected for study.

Growth conditions: The cells were grown aerobically at 30°C in a New Brunswick gyrorotary water bath. Growth was followed by reading the optical density of the cells at 520 nm. The optical density values ranging from 0.4 to 0.8 were selected corresponding to the log phase of cellular growth.

Cell breakage and sub cellular fractionation : A highly concentrated suspension of cells was prepared in 3.5 ml MTE buffer (0.25 M Mannitol, 0.05 M Tris-acetate buffer, pH 7.5 and 1mM EDTA, pH 7.2). The cells were broken in screw cap test tubes, containing glass beads (0.45 - 0.5 mm diameter), by vigorously shaking the mixture on a vortex genie mixer for 2 and a half minutes. The cells were chilled at intervals of 30 seconds. Cell extracts were obtained by centrifuging the homogenate at 3000 rpm for 10 minutes and saving the supernatant fluid. The mitochondrial particles were obtained from the cell extract by centrifuging the 3000 rpm supernatant at 15,000 rpm for 20 minutes. The mitochondrial pellet obtained was resuspended in 0.3 - 0.5 ml of MTE buffer to give a final protein concentration of 2-3 mg/ml.

In the case of δ-aminolevulinate debydratase assay, the homogenate was centrifuged at 17,000 rpm for 30 minutes to remove most of the cellular particles. The supernatant obtained was used for δ- aminolevulinate dehydratase measurements.

Respiration measurements: Oxygen uptake was measured by the method of Sherman, Fink and Lukins (140) with the following exceptions: Early to mid-log phase cells were used exclusively, samples of cells were washed and resuspended in cold 0.05 M potassium phosphate buffer, pH 6.8. Oxygen uptake of aliquots containing 0.6-2.0 mg cells was determined using a YSI model 53 oxygen electrode with 3% glucose added as the carbon source. Respiration rates were calculated by the method of Lessler (141) and are expressed as μl O_2/mg/hour.

Enzymatic assays: All enzyme assays were performed in a Beckman DB spectrophotometer (Model 25) at room temperature. The enzymatic assays were performed in triplicate and the standard deviation was from 5-10% on samples run on different days. The only exceptions were cytochrome c oxidase and NADH: cytochrome c reductase which showed a variation of as high as 20-25% from day-to-day.

a) Enzymes of the respiratory chain

(i) Succinate dehydrogenase was measured by the modified method of Kim and Beattie (55). The rate of reduction of 2-6 dichlorophenol indophenol was measured at 600 nm after addition of 0.1-0.2 mgs of mitochondrial protein to the cuvette containing 1 ml of 1mM dichlorophenol indophenol, 1.2 mM phenazine methosulfate, 1.5 mM KCN, 0.05 M phosphate buffer (pH 7.6) and 9mM succinate. The absorbance coefficient of 21 $mM^{-1}cm^{-1}$ was used.

(ii) NADH dehydrogenase was measured by the method of Kim and Beattie (55). The decrease in absorbance at 420 nm was measured after addition of 40-50 µgms of mitochondrial protein to a 1 ml cuvette containing 1mM potassium ferricyanide 0.1M tris buffer (pH 7.4), 10 µgms of antimycin A solution (prepared in ethanol), and 0.25 mM NADH. The absorbance coefficient of 1.0 $mM^{-1}cm^{-1}$ was used.

(iii) NADH and Succinate: cytochrome c reductase: The rate of reduction of cytochrome c was monitored at 550 nm for both the enzymes. The reaction was started by addition of 25-100 µgs of mitochondrial protein to the cuvette containing 100 µgs cytochrome c, 0.9 ml phosphate buffer, pH 7.2 (0.05M sodium phosphate, 2mM EDTA), 2 µgs KCN and either 0.4 mgs of $NADH_2$ (142) or 9 mmoles of Na_2 succinate (68) in 1 ml reaction mixture. The absorbance coefficient of 18.5 $mM^{-1}cm^{-1}$ was used.

(iv) Cytochrome c oxidase: was assayed according to the method of Smith (143). Reduced cytochrome c was prepared by addition of sodium dithionate to give a pale orange colour. The rate of oxidation of cytochrome c was followed at 550 nm after addition of 25-100 µgms of mitochondrial protein to the cuvette containing 80 µgs of reduced cytochrome c, 0.9 ml of phosphate buffer (0.05 M sodium phosphate and 2 mM EDTA, pH 7.2) in 1 ml reaction mixture. The absorbance co-efficient of 18.5 $mM^{-1}cm^{-1}$ was used.

b) Enzyme of heme synthesis

δ-aminolevulinate dehydratase was measured by the method of Nandi, Baker-Cohen and Shemin (144) as subsequently modified by Mahler and Lin (61). The amount of porphobilinogen formed was measured at 555 nm. The 2 ml reaction mixture containing 300 μmoles tris (hydroxymethyl) aminomethane buffer (pH 8.5), 150 μmoles KCl, 15 μmoles β-meracaptoethanol, 1.6 mg-3.2 mgs of cytosolic protein and 10 μmoles of δ-aminolevulinic acid neutralized with tris to pH 8.5, was incubated at 37°C for 60 minutes. The reaction was stopped by addition of 0.4 ml of 20% Trichloroacetic acid containing 0.1 M $HgCl_2$. The mixture was centrifuged at 5000 rpm for 10 min. and 1 ml of modified Ehrlich's mercury reagent was added to 1 ml of the supernatant. The absorbance was read at 555nm after 15 min. The amount of porphobilinogen formed was read against a standard porphobilinogen chart.

All samples were run at least four times and the values reported are an average of these values. A standard deviation of 20 - 25% was observed in the numbers from day-to-day. The amount of porphobilinogen formed was shown to be linear with time and the amount of cytosolic protein added.

c) Enzyme of citric acid cycle

Citrate synthase was assayed according to the method of Srere et. al (145). The reaction monitors the rate of acetyl-CoA cleavage. The increase in absorbance was monitored at 412 nm after addition of freshly prepared

2.3 mmoles oxaloacetic acid to 1 ml cuvette containing 0.2 mmole 5,5' - Dithiobis (-2-nitrobenzoic acid) prepared in 2% sodium bicarbonate and 0.4 - 0.6 mgms of the mitochondrial protein. The absorbance coefficient of 13.6 $nM^{-1}cm^{-1}$ was used.

d) Enzymes of carbohydrate metabolism

i) Galactokinase assay measures the phosphorylation of ^{14}C galactose (146). The procedure utilized to prepare the cells for measurements of enzyme activity is different from the one described previously and is therefore described below:

Preparation of cells: The procedure followed is that of Adams (74). The cells were grown overnight to log phase in 5 ml of YP + the appropriate sugar(s) and harvested. The cells are resuspended in 0.5 ml of 40% dimethyl sulfonate (DMSO) and then incubated in a water bath shaker at 30°C for 20 min. 4.5 ml of cold 0.1 M tris buffer, pH 8.0 was added to the cells, mixed and the cells were then centrifuged. The cells were next washed twice with tris buffer and finally suspended in 5.0 ml of this buffer. The optical density of the cell suspension at 650 nm served as a measure of cell density.

Assay: 50 µl of the DMSO treated cells were added to 50 µl of prewarmed galactokinase assay media (3 mM ^{14}C galactose, 3 mM ATP, 8mmM $MgCl_2$, 2 mM dithiothreitol in 1 mM tris-HCl, pH 8.0). 20 µl of the sample is immediately removed from the reaction mixture and spread on a Whatman DE 81 (2.5 cm diameter) filter paper. This serves as the blank. After 30 min

incubation period, 20 µl of the sample is removed from the reaction vial and spread on the DE81 filter paper. After drying for about 30 sec. the DE81 papers are put in 80% ethanol. Each (DE81) filter paper is then washed 3 times with 5 ml of water to remove unphosphorylated ^{14}C-galactose. After drying, the papers are placed in the scintillation vial containing the scintillant (toluene and omnifluor) and amount of radioactivity determined. The enzyme activity is reported as the nmoles of galactose-1-phosphate produced/min/O.D. 650 of the cells. The number of counts per minute was converted to nmoles of galactose-1-phosphate by dividing the number of cpm by nmoles of galactose in the assay media.

(ii) Maltase activity was measured by following the rate of splitting of p-nitrophenyl α-glucoside (PNPG) (colorless) to p-nitrophenol (yellow). 0.4-0.6 mgs. of cellular protein was added to the reaction mixture containing 0.1 gms p-nitrophenyl α-glucoside, 0.5 ml 20 mM dithiothreitol, 0.05 M phosphate buffer, pH 6.8 (161, 162). The reaction mixture was incubated and timed at 30°C in a water bath until yellow color appeared. The reaction was then stopped by addition of 2.0 ml of Na_2CO_3 and optical density read at 400 nm. One unit of maltase catalyzes the hydrolysis of 1.0 nmol of PNPG in 1 min.

(iii) α-Galactosidase activity was measured according to the method of Kew and Douglas (95). The amount of p-nitrophenol liberated upon hydrolysis of p-nitrophenyl-α-D galactopyranoside (PNPGal) was measured

at 400 nm. Assays were performed in 5.0 mM PNPGal buffered at pH 4.0 with 39 mM KP_2 PO_4 and 31 mM citric acid. Since α-galactosidase is an extracellular enzyme (163), assays were performed with whole cells. The reaction was started by addition of whole cells to the assay reagent and 0.1 ml samples were transferred to 0.9 ml Na_2 CO_3 and the optical density read at 400 nm. One unit of α-galactosidase is that amount capable of hydrolyzing 1.0 nmol of PNPGal per min under described conditions.

Protein determination: Protein was measured by the method of Lowry et. al. (160) using bovine serum albumin as the standard.

Matings: The parent strains are streaked on YPD plates and replicated on SD + adenine, wherever the selection of diploids was possible. If not, the plates were replicated on fresh YPD plates and allowed to grow for 2 days at 30°C. The diploids were then transferred on to sporulation medium and allowed to sporulate for 4-5 days.

The dissection on the diploids was carried out using Lawrence Precision dissecting microscope. The germination rate of the dissected spores was about 70-80%.

Chemicals and media: All the chemicals were obtained from Sigma Chemical Co. Saint Louis, Missouri, with the exception of the following. Sodium dithionate and glacial acetic acid from Fisher chemical Co. Fair Lawn, N.J., perchloric acid (from Dr. J. Berech, Biology Dept.

Queens College, Flushing, N.Y.) and $HgCl_2$ (from Mr. T. Hayden, Chemistry Dept., Queens College, Flushing, N.Y.).

Yeast extract, bactopeptone, bactoagar, bacto-glucose and synthetic minimal media were purchased from Difco Laboratories, Detroit, Michigan.

CHAPTER 3

BIOCHEMICAL CHARACTERIZATION OF GLUCOSAMINE RESISTANT MUTANTS

RESULTS

The study of glucose repression involves isolation of mutants that are altered in their regulation by glucose. A number of glucose analogues such as 2-deoxyglucose and D-glucosamine have been studied to determine their effectiveness in producing repression similar to that of glucose (112,113) with the aim of using them for isolating glucose repression insensitive mutants. Both 2-deoxyglucose (to a lesser extent) and D-glucosamine were found to produce such repression in yeast cells. Using D-glucosamine, the kinetics of repression and derepression of some mitochondrial enzymes and maltose, galactose fermentation enzymes was found to be similar to that of glucose (113). Since 2-deoxyglucose (which is phosphorylated and converted to UDP-deoxyglucose) (158) and D-glucosamine (only phosphorylated) (159) gave similar repression patterns to glucose, it suggested that it is either glucose or a phosphorylated derivative of glucose that is the controlling molecule in glucose repression. 2-deoxyglucose has been implicated in causing cell wall related changes, we have therefore used D-glucosamine as a gratuitous repressor to isolate 'glucose repression' resistant mutants.

Wild type yeast cells are unable to grow on lactate in the presence of glucosamine because glucosamine represses mitochondrial function (113). However, mutants can be isolated that are capable of growth on lactate in the presence of

glucosamine. Among such glucosamine resistant mutants, one should be able to identify mutants that exhibit reduced sensitivity to glucose for mitochondrial functions. Such class of mutants have been used for the purpose of present study.

Two parent strains have been used : M18-19F and M18-23E. The two strains are random spores from one cross. The genetic background could therefore be non-identical. While both these strains are sensitive to glucosamine, M18-23E is a little less sensitive to glucose repression than M18-19F. Table 1 shows the rates of oxygen uptake (QO_2) value of 27% on 5% glucose. This value closely corresponds with the derepression values obtained with other wild type strains used in our laboratory. M18-23E shows a derepressed value of oxygen uptake higher than M18-19F and therefore has been used in isolating glucosamine resistant mutants, in addition to M18-19F.

The parent strains M18-19F and M18-23E were mutagenized with nitrous acid (HNO_2) and glucosamine resistant mutants were obtained by selecting on YP + 2% lactate + 0.1 % glucosamine. Eight glucosamine resistant mutants were obtained from the M18-19F parent and eleven glucosamine resistant mutants were obtained from the M18-23E parent. The initial screening of the mutants involved the measurements of oxygen consumption following growth under repressed and derepressed conditions. All the mutants obtained from M18-19F parent showed QO_2 values similar to that of the parent, based on a comparison of the

Table 1. The rate of oxygen consumption of glucosamine resistant mutants. The log phase cells were suspended in medium containing 0.6 M glucose and the rate of oxygen uptake was measured using a YSI model 53 oxygen electrode. The activity is reported as μl O_2/mg of cells/hr.

TABLE 1

Strain	Derepressed QO_2 (2% Lactate Grown)	Repressed QO_2 (5% glucose Grown)	$\frac{\text{Repressed } QO_2}{\text{Derepressed } QO_2} \times 100$
M18-19F	54.7	14.7	27
M18-23E	50.9	26.6	52
GSA^r-1	54.1	40.7	75
GSA^r-4	46.0	36.5	80
GSA^r-5	41.0	39.0	95
GSA^r-11	53.9	42.4	79

repressed and derepressed ratios of O_2 consumption. However, four mutants obtained from the M18-23E parent showed higher values of O_2 consumption suggesting some degree of insensitivity of the respiratory chain to glucose repression. These values are shown in Table 1. Two of these four mutants, GSA^r-4 (for glucosamine resistance) and GSA^r-5 have been studied in detail.

The resistance to glucosamine seemed to be associated with a partial derepression of the respiratory chain. This suggested that the enzyme complexes involved in O_2 uptake, namely the enzymes of the electron transport chain and that of citric acid cycle (or only some enzymes from these groups) may be derepressed or exhibit partial insensitivity to glucose. For this reason biochemical assays for various respiratory enzymes were performed on these mutants.

The biochemical assays are also performed on 25A (glucosamine resistant spore) and 8C (glucosamine sensitive spore). These are spores obtained by a series of backcrosses to M18-23E. This backcrossing process will be described in detail in the next section, but, for present purposes 25A and 8C should be considered isogenic to GSA^r-4 at better than 98% of the loci except for the mating type locus, certain nutritional markers and the glucosamine resistance loci.

The results of succinic dehydrogenase and succinic: cytochrome c reductase assays are given in Table 2. The repression ratio is expressed as the percentage of the enzyme activity found under repressed conditions (5% glucose) as compared to the activity found under derepressed conditions

Table 2. The specific activities of Succinic dehydrogenase (A) and Succinate: cytochrome c reductase (B) of mitochondrial suspensions obtained from cells grown on 2% lactate and 5% glucose. The specific activity for succinic dehydrogenase is reported as (Δ O.D.$_{600}$/min/mg mitochondrial protein) x 100 and for succinate: cytochrome c reductase as μM cytochrome c reduced/min/mg mitochondrial protein.

TABLE 2

A. Succinic dehydrogenase

Strain	Derepressed 2% lactate	Repressed 5% glucose	$\frac{\text{Repressed}}{\text{Derepressed}} \times 100$
M18-19F	32.0	3.0	9
M18-23E	38.0	3.6	9
GSA^r-4	35.o	4.0	11
GSA^r-5	32.0	4.3	13
25A	31.0	4.7	15

B. Succinate: cytochrome c reductase

Strain	Derepressed 2% lactate	Repressed 5% glucose	$\frac{\text{Repressed}}{\text{Derepressed}} \times 100$
M18-19F	0.243	0.011	4
M18-23E	0.2	0.027	13
GSA^r-4	0.38	0.051	13
GSA^r-5	0.429	0.051	11
25A	0.312	0.023	7

(2% lactate). The parent strains exhibit a repression ratio of 9% for succinic dehydrogenase. A value of only 11%, 13% and 15% was observed for GSA^r-4 and GSA^r-5 and 25A respectively. This indicates that the glucosamine resistant mutants exhibit about the same sensitivity to glucose repression of succinic dehydrogenase as the parent strains. In the case of succinic: cytochrome c reductase, the two parent strains M18-19F and M18-23E exhibit a repression ratio of only 4% and 13% respectively. Comparison of these values to repression ratios of GSA^r-4, GSA^r-5 and 25A reveal no difference between the parent strains and the mutants. Based on these observations it is concluded that the phenotype observed in the glucosamine resistant mutants is not associated with the glucose insensitivity of the succinic dehydrogenase and succinic : cytochrome c reductase activities.

The next step involved in the study of these mutants was to assay for NADH : cytochrome c reductase and cytochrome c oxidase activities. Table 3 shows the repressed and derepressed levels of NADH : cytochrome c reductase and cytochrome oxidase in the two parents M18-19F, M18-23E and in GSA^r-4, GSA^r-5, 25A and 8C. The M18-19F parent exhibits a repression ratio of 24% and 31% for cytochrome c oxidase and NADH : cytochrome c reductase respectively. The M18-23E parent has however slightly elevated levels for cytochrome c oxidase, 38%, and NADH : cytochrome c reductase, 54%. The latter value indicates less than 50% repression of this enzyme as compared to fully induced levels on 2% lactate. The two mutants show

Table 3. The specific activities of NADH: cytochrome c reductase (A) and cytochrome c oxidase (B) of mitochondrial suspensions obtained from cells grown on 2% lactate and 5% glucose. The specific activity for NADH: cytochrome c reductase is reported as μM cytochrome c reduced/min/mg mitochondrial protein and for cytochrome c oxidase as μM cytochrome c oxidized/min/mg mitochondrial protein.

TABLE 3

A. NADH: cytochrome c reductase

Strain	Derepressed 2% lactate	Repressed 5% glucose	$\frac{\text{Repressed}}{\text{Derepressed}}$ x 100
M18-19F	1.20	0.376	31
M18-23E	1.08	0.582	54
GSA^r-4	1.02	0.94	92
GSA^r-5	0.916	0.992	+ 100
25A	0.954	0.86	90
8C	1.00	0.586	58

B. Cytochrome c oxidase

Strain	Derepressed 2% lactate	Repressed 5% glucose	$\frac{\text{Repressed}}{\text{Derepressed}}$ x 100
M18-19F	2.11	0.515	24
M18-23E	2.03	0.768	38
GSA^r-4	2.20	1.38	64
GSA^r-5	2.05	1.44	70
25A	2.20	1.39	63
8C	2.25	0.729	32

repression ratios for cytochrome c oxidase to be 64% in GSA^r-4 and 70% in GSA^r-5. These values are significantly higher than the parental values. Also, NADH : cytochrome c reductase levels are distinctly higher under repressed conditions in the two mutants. A repression ratio higher than 90% is observed in the mutants. Thus, GSA^r-4 and GSA^r-5 seem to be insensitive to glucose repression for NADH: cytochrome c reductase and cytochrome c oxidase activities.

The next enzyme to be studied was NADH dehydrogenase, a primary dehydrogenase in the electron transport chain. The values of NADH dehydrogenase activities are shown in Table 4. M18-19F and M18-23E show repression to about 60% of the lactate grown levels. This is not a marked repression. GSA^r-4 and GSA^r-5 do not show any significant difference from the parent M18-23E.

Based on these observations it seems that the insensitivity of the respiratory chain to glucose repression in our mutants, as examplified by high values of oxygen uptake on 5% glucose, is a direct result of the derepression of a portion of the electron transport chain. This portion includes complex I + III (NADH : cytochrome c reductase) and complex IV (cytochrome c oxidase).

In order to establish whether the glucosamine resistant phenotype is correlated with derepression of cytochrome c oxidase and NADH: cytochrome c reductase, a number of glucosamine resistant and glucosamine sensitive spores were selected at random from crosses between 25A (glucosamine resistant spore

from GSA^r-4) to M18-23E and GSA^r-5 to 8C (Dissections are shown in Table 8 of chapter 4), and assayed for both cytochrome c oxidase and NADH: cytochrome c reductase. The data obtained is shown in Table 5. It is seen that each time a spore has the glucosamine resistance phenotype it also exhibits glucose insensitivity of these two enzyme complexes. Correspondingly, the sensitive spores have levels similar to that of the parent M18-23E. Thus, either both phenotypes result from a pleiotropic mutation (or mutations) or the gene(s) for glucosamine resistance is strongly linked to gene(s) for glucose insensitivity of electron transport.

The activity of the enzyme involved in heme biosynthesis, δ-aminolevulinate dehydratase has been investigated. Table 6 shows the activity of this enzyme. M18-19F exhibits a repressed ratio of 23%. M18-23E, however, shows an unusually high repression ratio of 65%. GSA^r-4 and GSA^r-5 show no altered levels for this enzyme when compared to the parent strain, M18-23E.

The enzyme citrate synthase catalyzes the condensation of acetyl-CoA with oxaloacetate to form citric acid. The citrate synthase reaction is the primary pacemaker step of the TCA cycle; its rate is determined by the availability of acetyl-CoA and oxaloacetate and by the concentration of succinyl-CoA, which competes with acetyl-CoA and inhibits citrate synthase (147). Citrate synthase is subject to repression by glucose. This enzyme is repressed to 5% of the lactate grown levels in M18-19F and 12% in the M18-23E parent (Table 6). GSA^r-4 and

Table 4. The specific activity of NADH dehydrogenase on mitochondrial suspensions obtained from cells grown on 2% lactate and 5% glucose. The specific activity is reported as Δ O.D.$_{420}$/min/mg mitochondrial protein.

TABLE 4

Strain	Derepressed 2% lactate	Repressed 5% glucose	$\frac{\text{Repressed}}{\text{Derepressed}}$ x 100
M18-19F	1.77	1.17	66
M18-23E	1.74	1.00	57
GSA^r-4	1.52	1.11	73
GSA^r-5	1.67	1.13	67

Table 5. NADH: cytochrome c reductase (A) and cytochrome c oxidase (B) activities on mitochondrial suspensions obtained from 5% glucose grown cells. The strain 4-X comes from 25A (GSA^r-4) x M18-23E cross and 5-X comes from GSA^r-5 x 8C cross. The values on 2% lactate are taken as constant for both the enzymes (1.2 for NADH: cytochrome c reductase and 2.18 for cytochrome c oxidase) to avoid error in the repression ratio. The last column (C) shows the phenotype of the spore. The specific activity for NADH: cytochrome c reductase is reported as µM cytochrome c reduced/min/mg protein and for cytochrome c oxidase as µM cytochrome c oxidized/min/mg mitochondrial protein.

TABLE 5

Strain	A. NADH: cytochrome c reductase on 5% glucose	B. Cyt c oxidase on 5% glucose	C. Glucosamine phenotype
M18-23E	0.582	0.768	$GSA^{sensitive}$
GSA^{r}-4	0.94	1.38	$GSA^{resistant}$
GSA^{r}-5	0.992	1.44	$GSA^{resistant}$
4-12A	0.75	1.36	GSA^{r}
4-2A	0.84	1.25	GSA^{r}
4-3C	0.81	1.30	GSA^{r}
4-5D	0.92	1.30	GSA^{r}
4-6D	0.44	0.856	GSA^{s}
4-3B	0.473	0.673	GSA^{s}
4-5A	0.4	0.8	GSA^{s}
5-2A	0.81	1.37	GSA^{r}
5-28A	0.73	1.4	GSA^{r}
5-24A	0.81	1.2	GSA^{r}
5-26A	1.02	2.23	GSA^{r}
5-19A	0.457	0.54	GSA^{s}
5-19C	0.742	0.734	GSA^{s}
5-32A	1.09	1.8	GSA^{r}
5-29D	0.91	2.03	GSA^{r}

GSA^r-5 exhibit a significant glucose insensitivity of this enzyme. However, 25A shows a repressed ratio of 13% which is similar to that in M18-23E. Thus, the higher values obtained in GSA^r-4 and GSA^r-5 are not required for glucosamine resistance.

Enzymes of carbohydrate metabolism

The mutants have also been assayed for the activities of some of the enzymes involved in carbohydrate metabolism. The results for the activities of maltase, galactokinase and α- galactosidase are shown in Table 7. Galactokinase is found to be totally repressed on 5% glucose grown cells in the parent as well as in the mutants. α-galactosidase is responsible for hydrolysis of disaccharide melibiose into galactose and glucose. The enzyme is subject to strong repression by glucose in yeast. α-galactosidase is found to be as strongly repressed in the mutants as in the parent. Also, maltase is as glucose sensitive in GSA^r-4 and GSA^r-5 as in M18-23E.

Table 6. δ-aminolevulinate dehydratase activity (A) and citrate synthase (B) activity on 2% lactate and 5% glucose grown cells. The δ - aminolevulinate dehydratase activity done on cellular extract is reported as nmoles PBG/hr/mg cytosolic protein. The citrate synthase activity done on mitochondrial suspensions is reported as Δ O.D.$_{412}$/min/mg mitochondrial proteins.

TABLE 6

A. δ - aminolevulinate dehydratase

Strain	Derepressed 2% lactate	Repressed 5% glucose	$\frac{\text{Repressed}}{\text{Derepressed}}$ x 100
M18-19F	0.634	0.147	23
M18-23E	1.16	0.761	65
GSA^r-4	0.95	0.6	63
GSA^r-5	1.11	0.659	60

B. Citrate synthase

Strain	Derepressed 2% lactate	Repressed 5% glucose	$\frac{\text{Repressed}}{\text{Derepressed}}$ x 100
M18-19F	0.126	0.007	5
M18-23E	0.101	0.014	12
GSA^r-4	0.116	0.063	54
GSA^r-5	0.115	0.044	38
25A	0.115	0.015	13

Table 7. The specific activities of enzymes of carbohydrate metabolism on 2% galactose, 5% glucose and 2% galactose + 5% glucose grown cells. The activity for galactokinase is reported as μM gal-1-phosphate/min/O.D.$_{650}$, for maltase as Δ O.D.$_{400}$/hr/mg cytosolic protein and for α-galactosidase as Δ O.D.$_{400}$/min/O.D.$_{650}$.

TABLE 7

Strain	Galactokinase Act.			Maltase Activity			α-Galactosidase Activity		
	2% gal (Derep)	2% gal+ 5% glu (Rep)	$\frac{\text{Rep}}{\text{Derep}}$ x100	2% gal (Derep)	5% glu (Rep)	$\frac{\text{Rep}}{\text{Derep}}$ x 100	2% gal (Derep)	2% gal+ 5% glu (Rep)	$\frac{\text{Rep}}{\text{(Derep)}}$ x 10
M18-19F	2.05	0	0	32.02	3.92	12	2.21	0.35	16
M18-23E	3.38	0	0	18.44	4.28	23	4.05	0.38	9
GSA^r-4	1.41	0	0	17.0	4.98	28	2.09	0.01	0.5
GSA^r-5	1.92	0	0	15.0	4.7	31	1.72	0.07	4

DISCUSSION

D-glucosamine (0.1%) has been found to produce a repression of enzyme synthesis similar to that of glucose repression (113). Based on this observation, and making use of it, we have isolated two mutants GSA^r-4 and GSA^r-5 (both from M18-23E) that are resistant to glucosamine and to glucose repression of certain electron transport enzymes. More specifically, the enzymes cytochrome c oxidase and NADH: cytochrome c reductase were found to exhibit glucose insensitive functions. The glucose insensitivity of these enzymes is correlated with the glucosamine resistant phenotype. The two phenotypes segregate together in crosses to glucose insensitive strains. Some of the other respiratory enzymes that have been assayed for in the mutants such as succinic dehydrogenase, succinic: cytochrome c reductase and NADH dehydrogenase do not exhibit any decreased sensitivity to glucose. Thus the regulatory change is specific for the cytochrome containing complexes of the electron transport chain.

Glucosamine resistant mutants have been isolated by Ball and co-workers (149, 150) but under significantly different conditions. These mutants were isolated on YP glycerol + glucosamine (rather than lactate + glucosamine that has been used in our work). From previous work done by Furst and Michels (113), it was shown that the effect of D-Glucosamine on cells grown on glycerol is different from that on cells grown on lactate. In lactate grown cells D-Glucosamine acts as a repressor of enzyme synthesis.

This may not be the case when the cells are grown on glycerol or ethanol. Further, no biochemical work was reported for these mutants and hence it is not known whether this glucosamine resistant phenotype was associated with the derepression of the mitochondrial enzymes or was a result of a change in the property of glucosamine uptake. Thus, our class of mutants should be treated separately from the mutants isolated by Ball et. al.

Since only the cytochrome-containing complexes of the electron transport chain exhibit an altered glucose sensitivity, we looked at the possibility of decreased sensitivity of heme synthesis. Previously, many studies (129-135) have implicated the synthesis of heme as a major step in the regulation of the cytochrome containing enzymes of the respiratory chain. Since δ - aminolevulinate dehydratase is considered to be the rate limiting step in heme biosynthesis (61), we have assayed for this enzyme in our mutants in hope of finding whether our mutants are also derepressed for this enzyme. However, GSA^r-4 and GSA^r-5 did not show any significant change in the glucose sensitivity for this enzyme as compared to the sensitivity in the parent strain. Hence, the glucose insensitivity of NADH: cytochrome c reductase and cytochrome c oxidase in the mutants does not result from altered glucose sensitivity of heme synthesis.

Interestingly, 'glucose repression' resistant mutants were only obtained in M18-23E parent which shows higher activity for δ-aminolevulinate dehydratase on 5% glucose than

M18-19F parent. Whether this is purely coincidental or whether higher activities in M18-23E were of any significance in the isolation of mutants is not known. If a higher activity of δ-aminolevulinate dehydratase is in fact responsible, the rate of heme synthesis can be seen as a rate limiting factor in the glucose repression of the cytochrome containing complexes of electron transport.

A number of conclusions can be drawn regarding the regulatory mechanisms controlling the various aspects of glucose metabolism in yeast. The enzyme citrate synthase has been found to be normally repressed in glucosamine resistant mutants. Since this enzyme is the first enzyme in the TCA cycle and it must be derepressed when the TCA cycle is operating, it is concluded that the TCA cycle is still normally glucose repressed in these mutant strains. Thus, an independent pathway exists in yeast cells for regulating the TCA cycle. Also, the fact that the TCA cycle enzymes appear to be normally repressed by glucose while the rate of oxygen consumption is partially glucose insensitive in these mutants implies that it is the activity of the electron transport chain which is rate limiting for oxygen consumption.

The enzymes NADH: cytochrome c reductase and succinic: cytochrome c reductase share a common segment of the respiratory chain at coenzyme Q and complex III which includes cytochromes $\underline{b}$ and $\underline{c}_1$ (3). We have observed that derepression of NADH: cytochrome c reductase can occur independently of derepression

of succinic : cytochrome c reductase and therefore these two enzyme complexes are probably regulated differently. Kim and Beattie (55) have also made a similar observation in that succinate : cytochrome c reductase and NADH : cytochrome c reductase responded differently to certain protein synthesis inhibitors and behave differently during derepression studies. Further, it was suggested that these differences could not be due to differences in the primary dehydrogenases such as NADH dehydrogenase or succinic dehydrogenase as they are not the rate limiting enzymes in determining the activities of NADH : cytochrome c reductase and succinic : cytochrome c reductase respectively. Therefore, these differences could be due to differences in the coenzyme Q pools (if there are separate pools for these two enzyme complexes) and/or differences in $\underline{bc}_1$ segment of complex III. However, recent studies by Brown and Beattie (148) show no such existence of separate coenzyme Q pools nor of cytochromes $\underline{b}$ and $\underline{c}_1$ for these enzyme complexes. Hence, both enzymes do share a common pool of coenzyme Q and also common segments of complex III but nevertheless, are regulated differently by glucose. Exactly how this may be achieved is not understood at present. It can only be hypothesized that this regulation may then occur at $\underline{bc}_1$ segment for these two enzyme complexes.

The enzymes of carbohydrate metabolism that have been assayed are maltase, galactokinase and α-galactosidase. All three enzymes are found to exhibit normal glucose repression and therefore are not a component of the regulatory system that has been derepressed in the glucosamine resistant mutants.

In conclusion therefore, we have identified mutant strains resistant to glucose repression for NADH: cytochrome c reductase and cytochrome c oxidase components of the respiratory chain. Other enzymes of the respiratory chain do not seem to be regulated by the mutation(s) present in GSA^r-4 and GSA^r-5. In the next chapter, a detailed genetic investigation has been carried out on GSA^r-4 and GSA^r-5 to identify the loci involved in producing the glucose insensitivity of these strains.

CHAPTER 4

GENETIC ANALYSIS OF GLUCOSE REPRESSION RESISTANT MUTANTS OF MITOCHONDRIAL BIOGENESIS

RESULTS

The results of the crosses between 25A x M18-23E and GSA^r-5 x 8C are shown in Table 8. As briefly mentioned in chapter 3, 25A is a glucosamine resistant spore while 8C is glucosamine sensitive spore which have been used in the present study as mater strains. These spores were obtained by a series of backcrosses to the parent M18-23E. To do this, GSA^r-4 was crossed to M18-19F and a spore from this cross, shown to be both glucosamine resistant and insensitive to glucose repression of the cytochromes, was backcrossed to the parent M18-23E. A spore from this cross with the desired phenotype (glucosamine resistant and glucose insensitive cytochrome synthesis) was again backcrossed to M18-23E. This was repeated for four more generations for a total of six backcrosses. The strains isolated from this process are, statistically speaking, otherwise, isogenic to M18-23E parent at better than 98.4% of the loci. 25A differs from M18-23E at the mating type locus, is adel trp5, glucosamine resistant and glucose insensitive for cytochrome synthesis. 8C, isolated from the final backcross to M18-23E, is like M18-23E but of opposite mating type (α adel ura3 and glucosamine sensitive).

The results shown in Table 8 are consistent with the hypothesis that the mutant phenotype 'glucosamine resistance' in both GSA^r-4 and GSA^r-5 involve two unlinked nuclear loci and the presence of mutant alleles at both loci is necessary for the glucosamine resistance phenotype.

Table 8. The results of tetrad dissections between 25A x M18-23E and GSA^r-5 x 8C.

TABLE 8

Tetrad Analysis of Glucosamine Resistant Mutants

Diploid	Ratio $GSA^{sensitive}$: $GSA^{resistant}$ in 4-spore Tetrads		
	2:2	3:1	4:0
25A x M18-23E	3	16	4
GSA^{r}-5 x 8C	5	24	6
Expected for a Dihybrid	1	4	1

Based on the segregation pattern obtained in Table 8 it is proposed that there are two nuclear loci, grc1 and grc2 (wild type GRC1 and GRC2) operating in GSA^r-4. Similarly, the loci involved in GSA^r-5 have been named grc3 and grc4 (wild type GRC3 and GRC4). (Later in the study it will be shown that different alleles of the GRC1 gene are present in GSA^r-4 and GSA^r-5).

According to the two gene segregation pattern for glucosamine resistance, as shown from the results of Table 8, it is proposed that in a tetrad with a 3 glucosamine sensitive: 1 glucosamine resistant spores, the resistant spore contains mutant alleles at both GRC loci. Further, one sensitive spore should have no grc mutation and exhibit a classical two gene segregation pattern when mated to a glucosamine resistant spore. The two remaining sensitive spores should each have one mutation namely, grc1 (or grc3 in GSA^r-5) and grc2 (or grc4 in GSA^r-5) and should give only 2 sensitive: 2 resistant type tetrads when crossed to a resistant spore.

Such crosses have been performed on tetrads obtained from 25A x M18-23E cross and the results are shown in Table 9. Tetrad #9 has one glucosamine resistant spore 9A and three sensitive spores 9B, 9C and 9D. 9A mated with another resistant spore 17C gives only glucosamine resistant progeny. 9A mated with 9B gives a two gene segregation pattern for glucosamine resistant phenotype suggesting that 9B is wild type at both GRC loci found in GSA^r-4, GRC1 and GRC2.

Table 9. The phenotype and the proposed genotypes of the 3 sensitive spores of tetrads #9 and #17 from 25A x M18-23E cross.

TABLE 9

Diploids	# of Tetrads	GSA^s: GSA^r in 4-spore tetrads 0:4	2:2	3:1	4:0	Proposed genotype in the sensitive spore
9A x 17C (GSA^r x GSA^r)	12	12	0	0	0	
9A x 9B (GSA^r)	17	0	2	11	4	GRC1 GRC2
9A x 9C (GSA^r)	17	0	17	0	0	grc1 GRC2
3B x 9D (GSA^r)	11	0	11	0	0	GRC1 grc2
17C x 17A (GSA^r)	8	0	8	0	0	GRC1 grc2
17C x 17D (GSA^r)	15	0	15	0	0	grc1 GRC2
5D x 17B (GSA^r)	11	0	2	7	2	GRC1 GRC2

9A mated with 9C gives tetrads that show $2GSA^s$: $2GSA^r$ phenotype indicating the presence of one grc gene. 9D mated with 3B (another GSA resistant spore from the same cross) gives tetrads that again show $2GSA^s$: $2GSA^r$ phenotype. Thus 9D also has one grc gene. The mutation in 9C has been named grc1 and in 9D has been named grc2. 9A has both grc1 and grc2 and hence glucosamine resistant phenotype. Similar observation has been made for tetrad #17 from 25A x M18-23E cross and the results are also shown in Table 9. Here 17C is the resistant spore and thus is genotypically grc1 grc2. 17B is wild type for both grc loci, GRC1 GRC2, since a 2 gene segregation pattern is obtained when it is crossed to a resistant spore. 17A and 17D both clearly each contain a single grc mutation; which GRC gene is mutant may be determined by crosses to the tester spores from tetrad #9.

Crosses between a spore 9C (of genotype grc1 GRC2) with spore 17D results in the progeny that are all glucosamine sensitive. The results of this cross are shown in Table 10. Thus, 17D is grc1 GRC2. However, cross between 9C (grc1 GRC2) and 17A results in a clear two gene segregation pattern for glucosamine resistance indicating that 17A must be GRC1 grc2. This again shows that both grc1 and grc2 must be present in a spore to produce the glucosamine resistant phenotype.

Further proof of this comes from the genetic crosses shown in Table 11. Both 6C and 6D spores have been randomly selected from tetrad #6 of 25A x M18-23E cross (shown in Table 8). This tetrad shows a $4GSA^s$: $0GSA^r$ phenotype and is therefore a

Table 10. The specificity of interaction between the grc1 and grc2 loci in GSA^r-4.

Table 11. The segregation of grc loci in a $4GSA^s$: $0GSA^r$ tetrad from 25A x M18-23E cross. For further explanation, see text.

TABLE 10

Diploid	# of Tetrads	Ratio GSA^{sensitive}: GSA^{resistant} in 4-spore tetrads		
		2:2	3:1	4:0
9C x 17D ($\frac{grc1}{GRC2}$ ($\frac{grc1}{GRC2}$)	7	0	0	7
9C x 17A ($\frac{grc1}{GRC2}$) ($\frac{GRC1}{grc2}$)	19	2	13	4

TABLE 11

Diploid	# of Tetrads	Ratio GSA^{sensitive}: GSA^{resistant} in 4 spore tetrads		
		2:2	3:1	4:0
6C x 3D (GSA^r)	8	8	0	0
6D x 3C (GSA^r)	9	9	0	0

non-parental ditype tetrad. Hence, theoretically each spore from this tetrad must have at least one grc mutant allele. Both 6C and 6D have been mated to glucosamine resistant spores and all the tetrads obtained from these crosses exhibit a $2GSA^r$: $2GSA^s$ phenotype proving that 6C and 6D carry a mutation in a single GRC gene.

In GSA^r-5, similarly it is proposed that the presence of grc3 and grc4 allows for the glucosamine resistant phenotype. Table 12 shows the segregation of these two gene loci in $1GSA^r$: $3GSA^s$ tetrad. 2A is a glucosamine resistant spore and therefore is mutant at grc3 and grc4. 2B (GSA^s) mated to 2A gives tetrads that are all $2GSA^r$: $2GSA^s$ phenotype. 2C mated to 2A (GSA^r) gives a two gene segregation pattern for glucosamine resistance. 2D mated to another glucosamine resistant spore 12B gives a $2GSA^r$: $2GSA^s$ phenotype in all the tetrads indicating a single gene difference. The mutation in 2B has been named grc3 and the mutation in 2D has been named grc4.

The genotype for tetrad #16 from the cross GSA^r-5 x 8C has also been determined and the results are shown in Table 12. 16B is mutant at grc3 and grc4 and is glucosamine resistant. As in GSA^r-4, only combinations between grc3 and grc4 allow for the glucosamine resistant phenotype and each mutation alone is not sufficient for growth on glucosamine. Crosses between spores of the genotype GRC3 grc4 (or between spores of the genotype grc3 GRC4) always yield glucosamine sensitive progeny. The results of this cross are shown in Table 13. The genotypes of the spores used in these crosses were established by mating to proper tester strains. Also, the cross between spores of the genotypes GRC3 grc4 x grc3 GRC4 produces resistant spores

Table 12. The phenotype and the proposed genotypes of the 3 sensitive spores of tetrads #2 and # 16 from GSA^r-5 x 8C cross.

TABLE 12

Diploids	# of Tetrads	GSA^s: GSA^r in 4-spore tetrads 2:2	3:1	4:0	Proposed genotype in the sensitive spore
2A x 2B (GSA^r)	13	13	0	0	grc3 GRC4
2A x 2C (GSA^r)	5	1	4	0	GRC3 GRC4
12Bx 2D (GSA^r)	12	12	0	0	GRC3 grc4
16B x 16A (GSA^r)	14	14	0	0	GRC3 grc4
16B x 16C (GSA^r)	9	1	6	2	GRC3 GRC4
2A x 16D (GSA^r)	11	11	0	0	grc3 GRC4

Table 13. The results of the crosses performed between 16A (grc4 GRC3) x 10A (grc4 GRC3), 4B (grc3 GRC4) x 2B (grc3 GRC4) and 16A (GRC3 grc4) x 2B (grc3 GRC4).

TABLE 13

Diploid		# of Tetrads	Ratio GSA^{sensitive} : GSA^{resistant} in 4-spore tetrads		
			2:2	3:1	4:0
16A (GRC3 grc4)	x 10A (GRC3 grc4)	10	0	0	10
4B (grc3 GRC4)	x 2B (grc3 GRC4)	7	0	0	7
16A (GRC3 grc4)	x 2B (grc3 GRC4)	10	1	7	2

with a two gene segregation pattern for glucosamine resistance.

All of the GRC mutations are nuclear genes as demonstrated by the various crosses described above. Additional proof that these genes are not mitochondrial comes from the cross performed between a glucosamine resistant spore, (18A, grc1 grc2) that had been subjected to ethidium bromide treatment sufficient to delete all or most of the mitochondrial DNA, and M18-23E. A two gene segregation pattern was observed in the progeny for glucosamine resistance. If glucosamine resistance phenotype was due to mitochondrial genes, all the progeny would have been glucosamine sensitive. Similar situation is found to exist for the mutations in GSA^r-5.

In the previous chapter, we have demonstrated that the glucosamine resistant phenotype segregates with the glucose insensitive synthesis of NADH: cytochrome c reductase and cytochrome c oxidase. We have postulated that a pleiotropic mutation (or mutations) are responsible for both phenotypes. Having shown that two mutations are present in both GSA^r-4 and GSA^r-5, and both mutations must be present for the glucosamine resistant phenotype to be expressed, the following question is posed. In GSA^r-4, which genotypically is grc1 grc2, is grc1 perhaps regulating the expression of NADH: cytochrome c reductase portion of the respiratory chain and grc2 regulating cytochrome c oxidase or vice versa? In order to answer this question, tetrads have been chosen that exhibit a $1GSA^r$: $3GSA^s$ phenotype and assays for both of these enzymes performed on all the four spores. The results of the assays done on tetrads # 12, # 9 and # 17 from 25A x M18-23E cross are shown

in Table 14. From the table, it is seen that only the resistant spore in each of the three tetrads has high levels for both NADH : cytochrome c reductase and cytochrome c oxidase enzymes on 5% glucose. None of the other sensitive spores bearing a mutation in only one grc locus shows any significantly altered levels for any of the two enzymes. The values for either of the enzymes is seen to be similar to that for the wild type parent, M18-23E. Hence, no correlation between the presence of a grc gene and derepression of a particular segment of the respiratory chain is observed.

Similarly, enzyme assays for NADH : cytochrome c reductase and cytochrome c oxidase activities have been conducted on tetrads #2 and #19 from GSA^r-5 x 8C. The results are shown in Table 15. Only 2A and 19B (both are glucosamine resistant) show high levels for the activity of these two enzymes on 5% glucose. None of the other sensitive spores from these two tetrads show any elevated levels for these two enzymes. Again, no correlation between the presence of grc mutation and derepression of a specific segment of the respiratory chain could be shown. Therefore, it is clear that mutant alleles must be present at both GRC1 and GRC2 in GSA^r-4 (and GRC3 and GRC4 in GSA^r-5) for the glucose insensitive synthesis of cytochrome oxidase and NADH: cytochrome c reductase and neither gene is able to express its phenotype independently of the other gene.

Table 14. NADH: cytochrome c reductase and cytochrome c oxidase activities on all 4 spores of the tetrads #12, #9 and #17 from 25A x M18-23E cross. The activities are measured on mitochondrial suspensions of 2% lactate and 5% glucose grown cells. The data for 2% lactate was found to be nearly identical for all spores (1.2 for NADH: cytochrome c reductase and 2.18 for cytochrome c oxidase) and therefore is not shown in the table. The specific activities for NADH: cyt c reductase are reported as μM cyt c reduced/min/mg protein and for cytochrome c oxidase as μM cyt c oxidized/min/mg mitochondrial protein.

TABLE 14

Spore	NADH:cyt c reduct on 5% glucose	Cyt c oxidase on 5% glucose	GSA phenotype	Genotype
12A	0.75	1.36	GSA^r	grc1 grc2
12B	0.42	0.786	GSA^s	grc1 GRC2
12C	0.64	0.745	GSA^s	GRC1 GRC2
12D	0.528	0.82	GSA^s	GRC1 grc2
9A	0.84	1.33	GSA^r	grc1 grc2
9B	0.44	0.82	GSA^s	GRC1 GRC2
9C	0.56	0.82	GSA^s	grc1 GRC2
9D	0.56	0.788	GSA^s	GRC1 grc2
17A	0.37	0.72	GSA^s	GRC1 grc2
17B	0.58	0.77	GSA^s	GRC1 GRC2
17C	0.937	1.30	GSA^r	grc1 grc2
17D	0.49	0.69	GSA^s	grc1 GRC2

Table 15. NADH: cytochrome c reductase and cytochrome c oxidase activities on all 4 spores of the tetrads #2 and # 19 from GSA^r-5 x 8C cross. The activities are measured on mitochondrial suspensions of 2% lactate and 5% glucose grown cells. The data for 2% lactate grown cells was found to be nearly identical for all spores (1.1 for NADH: cytochrome c reductase and 2.2 for cytochrome c oxidase) and therefore is not shown in the table. The specific activities for NADH: cytochrome c reductase are reported as μM cyt c reduced/min/mg protein and for cytochrome c oxidase as μM cyt c oxidized/min/mg mitochondrial protein.

TABLE 15

Spore	NADH:cyt c reductase on 5% glucose	Cyt c oxidase on 5% glucose	GSA phenotype	Genotype
2A	0.81	1.37	GSA^r	grc3 grc4
2B	0.65	0.82	GSA^s	grc3 GRC4
2C	0.64	0.86	GSA^s	GRC3 GRC4
2D	0.95	0.75	GSA^s	GRC3 grc4
19A	0.45	0.54	GSA^s	GRC3 GRC4
19B	1.0	2.26	GSA^r	grc3 grc4
19C	0.742	0.734	GSA^s	grc3 GRC4
19D	0.83	0.65	GSA^s	GRC3 grc4

The dominant or the recessive character of the grc mutation is shown in Table 16. Both the grc1 and grc2 genes are partially dominant as seen from the increased levels of both cytochrome c oxidase and NADH : cytochrome c reductase in the diploid heterozygotes GSA^r-4 x 8C (grc1/GRC1 grc2/GRC2) and 25A x M18-23E (grc1/GRC1 grc2/GRC2). Also grc3 and grc4 in GSA^r-5 are found to be partially dominant in the heterozygotes GSA^r-5 x 8C (grc3/GRC3 grc4/GRC4). The cytochrome values have also been checked for the diploid between 25A x GSA^r-5 (grc1 / grc4 grc2/grc3). Again, the genes are only partially dominant for cytochrome c oxidase and seem to be fully derepressed for the activity of NADH : cytochrome c reductase.

Complementation tests were done in order to determine if grc mutations generated in GSA^r-4 and GSA^r-5 are allelic to each other. Three independent diploids were obtained by mating 25A (grc1 grc2) with GSA^r-5 (grc3 grc4), GSA^r-4 with 6B (grc3 grc4) and GSA^r-5 with 10B (grc1 grc2) and the phenotype of the diploids checked. All the diploids were found to be sensitive. A high degree of mitotic recombination was observed in the diploids which gave rise to a large number of resistant colonies against a sensitive background. If the two genes were allelic to each other, the diploids would have been resistant. Tetrad analysis was also performed on the three diploids and the results are shown in Table 17. The segregation of glucosamine resistance phenotype in the tetrads depends upon the allelic nature and the interaction between

Table 16. The cytochrome c oxidase and NADH: cytochrome c reductase activity of diploids between the glucosamine resistant mutants and the parent. The activity for cytochrome c oxidase is reported as μM cytochrome c oxidized/ min/mg mitochondrial protein and for NADH: cytochrome c reductase as μM cytochrome c reduced/ min/mg mitochondrial protein.

TABLE 16

A. Activity of cytochrome c oxidase

Diploid	Derepressed Activity (2% lactate grown)	Repressed Activity (5% glucose grown)	$\frac{\text{Repressed}}{\text{Derepressed}}$ x 100
M18-23E x 8C	2.3	0.823	35
GSA^r-4 x 8C	2.4	1.31	54
GSA^r-5 x 8C	2.4	1.12	46
25A x M18-23E	2.35	1.27	54
25A x GSA^r-5	2.4	1.2	50

B. Activity of NADH: cytochrome c reductase

Diploid	Derepressed Activity 2% lactate grown)	Repressed Activity (5% glucose grown)	$\frac{\text{Repressed}}{\text{Derepressed}}$ x 100
M18-23E x 8C	1.03	0.587	57
GSA^r-4 x 8C	0.95	0.912	96
GSA^r-5 x 8C	1.15	0.794	70
25A x M18-23E	0.95	0.742	78
25A x GSA^r-5	1.08	0.928	86

the grc loci. Some of these cases will be listed below in order to follow the pattern of glucosamine resistance in the dissected diploids :

CASE I : The grc mutations in GSA^r-4 and GSA^r-5 are allelic to each other. This should result in all tetrads of the parental kind. All the four spores of the tetrad should be glucosamine resistant resulting from a homozygous diploid grc1/grc1 grc2/grc2.

CASE II : The grc mutations in GSA^r-4 and GSA^r-5 are non-allelic and only the combinations grc1 grc2 (as in GSA^r-4) and grc3 grc4 (as in GSA^r-5) result in a glucosamine resistant phenotype. This would result in generation of higher number of tetrads that show $2GSA^r$: $2GSA^s$ phenotype. Other tetrads that exhibit $3GSA^r$: $1GSA^s$, $1GSA^r$: $3GSA^s$, $4GSA^r$: $OGSA^s$ and $OGSA^r$: $4GSA^s$ will also be generated but their proportion will be very low.

CASE III : The grc mutations in GSA^r-4 and GSA^r-5 are non-allelic and there is a specific interaction between the various grc mutations. grc1 can combine with grc2 (as in GSA^r-4) and with either grc3 or grc4, but not both, to produce the GSA^r phenotype. Also, grc3 can combine with grc4 (as in GSA^r-5) and either with grc1 or grc2, but not with both, to produce the GSA^r phenotype. For example, only the following combinations will produce the GSA^r phenotype: grc1 grc2, grc1 grc3 or grc1 grc4, grc3 grc4, and grc3 grc1 or grc3 grc2. This will result in the following segregation for glucosamine resistance 6(4 GSA^r : O GSA^s) : 72 (3 GSA^r : 1 GSA^s): 109 (2 GSA^r:2GSA^s) : 28 (1 GSA^r : 3 GSA^s) : 1(O GSA^r :4 GSA^s). In short there will be about 1½ as many

Table 17. Tetrad analysis of GSA^r-4 x GSA^r-5. Three independent diploids were obtained from the crosses GSA^r-4 x 6B (of the same genotype as GSA^r-5), GSA^r-5 x 10 B (of the same genotype as GSA^r-4) and 25A x GSA^r-5. Dissections were performed and only 4 spored asci that segregated 2:2 for a nutritional marker were selected.

TABLE 17

Tetrad Analysis of GSA^r-4 x GSA^r-5

Diploid	# of Tetrads	Ratio $GSA^{sensitive}$: $GSA^{resistant}$ in 4-spore tetrads			
		3:1	2:2	1:3	0:4
GSA^r-4 x 6B	15	0	8	5	2
GSA^r-5 x 10D	14	1	11	3	0
25A x GSA^r-5	26	0	8	15	3

2 GSA^r : 2GSA^s tetrads than the 3 GSA^r :1 GSA^s tetrads. Also, the proportion of the 4 GSA^r : O GSA^s tetrads will be very low.

CASE IV : The grc mutations in GSA^r-4 and GSA^r-5 are non-allelic and the presence of any two grc mutations in a haploid spore can result in a glucosamine resistant phenotype, e.g. grc1 can interact with either grc4, grc3 or with grc2 to give a glucosamine resistant phenotype. This will follow a 4 gene segregation pattern which has been calculated to give a ratio of 4.6 (2GSA^r : 2GSA^s) : 8.8 (3GSA^r : 1GSA^s) : 1(4GSA^r : OGSA^s). In short, there will be twice as many tetrads with a 3GSA^r : 1GSA^s phenotype as with the 2GSA^r : 2GSA^s phenotype.

CASE V : One gene from GSA^r-4 is allelic with one in GSA^r-5. Here glucosamine resistance follows that of a two gene segregation pattern, i.e. 1(4GSA^r : O GSA^s) : 4(3 GSA^r : 1 GSA^s) : 1(2 GSA^r : 2 GSA^s).

The results of dissections of crosses between resistant spores from GSA^r-4 and GSA^r-5 are shown in Table 17. All the three crosses should be, genetically speaking, identical. The spores 6B and 10C are glucosamine resistant and were obtained from the crosses GSA^r-5 x 8C and 25A x M18-23E respectively (shown in Table 8). The cross between 25A x GSA^r-5 follows the pattern for two gene segregation more closely than for any other category while the two other crosses follow a different pattern (the presence of one 1 GSA^r :3 GSA^s tetrad can also be explained by gene conversion). These dissimilar results can probably result

from the sampling error as the number of tetrads dissected is small in the latter two cases. While the results in Table 17 show that case I is not true, no additional conclusions can be made.

In order to decide between cases II, III and IV, the following crosses between spores containing a single grc mutation were done. 17D (grc1 GRC2, Table 9) was crossed with 2B (grc3 GRC4, Table 12) and 2D (GRC3 grc4, Table 12). Also, another spore 22A (genotypically GRC1 grc2 as determined by crosses to known tester strains)was crossed with 2B (grc3 GRC4) and 2D (GRC3 grc4). The results of these crosses are shown in Table 18. They indicate that only specific combinations of the types grc1 grc3 and grc2 grc4 result in a glucosamine resistant phenotype. The results also indicate that the grc mutations are non-allelic. The mutation at grc1 locus can interact only with grc3 (and not with grc4) to produce a glucosamine resistant phenotype. Similarly, the mutation at grc2 locus can interact only with the mutation at the grc4 locus (and not with grc3). This specificity suggests that grc1 and grc4 loci are regulating functions, such as the same segment of the electron transport chain, while grc2 and grc3 are regulating another aspect of the electron transport chain.

In order to determine if any of the grc mutations in GSA^r-4 are allelic with any grc mutation in GSA^r-5, a different set of crosses was devised. Diploids were obtained from spores containing grc mutations in the following combinations: 3A (grc1 grc3) was mated with 15A (grc3 grc4) and these diploids were found to be resistant indicating grc1 and grc4 to be

Table 18. The specificity of interaction between the grc mutations in GSA^r-4 and GSA^r-5. Diploids were obtained between 17D (grc1 GRC2) x 2B (grc3 GRC4), 17D (grc1 GRC2) x 2D (GRC3 grc4), 22A (GRC1 grc2) x 2B (grc3 GRC4) and 22A (GRC1 grc2) x 2D (GRC3 grc4). Dissections were performed on these tetrads and only 4 spored asci that segregated 2:2 for a nutritional marker were selected.

TABLE 18

Diploid		Ratio GSA^{sensitive} : GSA^{resistant} in 4-spore tetrads		
	# of Tetrads	2:2	3:1	4:0
17D x (grc1 / GRC2)	2B (grc3 / GRC4)	15	4	8	3
17D x (grc1 / GRC2)	2D (GRC3 / grc4)	12	0	0	12
22A x (GRC1 / grc2)	2B (grc3 / GRC4)	15	0	0	15
22A x (GRC1 / grc2)	2D (GRC3 / grc4)	14	3	8	3

allelic. (Spore 3A was obtained from the cross 17D x 2B, shown in Table 18; spore 15A was obtained from the cross GSA^r-5 x 8C, shown in Table 8). However, the diploid between GSA^r-5 (grc3 grc4) x 6C (grc2 grc4) was found to be sensitive implying that grc2 and grc3 are not allelic (spore 6C was obtained from the cross 22A x 2D, shown in Table 18).

The activities for both cytochrome c oxidase and NADH: cytochrome c reductase were measured on the (3A x 15A) and (GSA^r-5 x 6C) diploids and the results are shown in Table 19. Comparison of the cytochrome values of the (GSA^r-5 x 6C) diploid with the heterozygous diploids (25A x M18-23E and GSA^r-5 x 8C, shown in Table 16) reveal no significantly elevated levels of cytochrome on 5% glucose, a result consistent with the hypothesis that grc2 and grc3 are not allelic. The situation, however, is complicated for the (3A x 15A) diploid. Here, even though this diploid is glucosamine resistant phenotypically, the cytochrome values are comparable to the heterozygous glucosamine sensitive diploids (25A x M18-23E and GSA^r-5 x 8C). These results lead us to believe that the grc mutations may also regulate other enzymes which have not been assayed for in the present study, but which are needed for the glucosamine resistant phenotype and which are not sufficiently derepressed in the heterozygotes to allow for resistant growth on lactate plus glucosamine.

The final proof of the hypothesis that grc1 and grc4 are in fact allelic while the grc2 and grc3 are not allelic comes

Table 19. The cytochrome c oxidase and NADH: cytochrome c reductase activities on mitochondrial suspensions obtained from the diploids between 3A x 15A and GSA^r-5 x 6C. The activity for cytochrome c oxidase is reported as μM cytochrome c oxidized/min/mg protein and for cytochrome c oxidase as μM cytochrome c reduced/min/mg protein. For further explanation see text.

TABLE 19

A. Activity of cytochrome c oxidase

Diploid	Derepressed Activity (2% lactate grown)	Repressed Activity (5% glucose grown)	$\frac{\text{Repressed}}{\text{Derepressed}} \times 100$
3A x 15A ($\frac{grc1}{grc3} / \frac{grc4}{grc3}$)	2.3	1.2	52
GSA^r-5 x 6C ($\frac{grc4}{grc2} / \frac{grc4}{grc3}$)	2.3	1.02	44

B. Activity of NADH: cytochrome c reductase

Diploid	Derepressed Activity (2% lactate grown)	Repressed Activity (5% glucose grown)	$\frac{\text{Repressed}}{\text{Derepressed}} \times 100$
3A x 15A ($\frac{grc1}{grc3} / \frac{grc4}{grc3}$)	0.72	0.623	86
GSA^r-5 x 6C ($\frac{grc4}{grc2} / \frac{grc4}{grc3}$)	0.788	0.63	80

from the following crosses. Tetrad dissections were performed on the (3A x 15A) and the (GSA^r-5 x 6C) diploids. The results are shown in Table 20. If grc1 and grc4 are mutations in different loci one would expect the progeny to exhibit a two gene segregation pattern for glucosamine resistance. However, if grc1 and grc4 are in fact allelic, all the progeny would be composed of 4 GSA^r : O GSA^s tetrads. This will result from a homozygous diploid grc1-1/grc1-4 grc3/grc3. Our results from Table 20 indicate that all tetrads obtained from this cross are of the $4GSA^r$: $OGSA^s$ phenotype. Therefore, it is implied that grc1 and grc4 are alleles of the same gene GRC1 and can therefore be represented as grc1-1 and grc1-4.

Having established that the mutations grc1 and grc4 are alleles of the same locus GRC1, the allele testing for grc2 and grc3 becomes relatively easy to perform. The dissection of the diploid between GSA^r-5 (which now becomes grc3 grc1-4) and 6C (which is grc2 grc1-4) is shown in Table 20. From our results it can be advanced that if grc2 and grc3 were allelic all the progeny would be of the glucosamine resistant type and since that is not the case, we conclude that grc2 and grc3 are not allelic. Also, the segregation of glucosamine resistance follows the pattern expected of two unlinked nuclear loci.

Therefore, it is concluded that grc1 and grc4 are allelic to each other and in combination with grc2 in GSA^r-4 and with grc3 in GSA^r-5 allow for the glucose insensitive functioning of the respiratory chain between NADH and oxygen

and possibly also for some other enzymes. The exact nature of these enzymes remains unknown at present.

Table 20. Complementation tests on grc mutations. The diploids 3A x 15A and GSA^r-5 x 6C were dissected and only 4 spored tetrads that segregated 2:2 for a nuclearly inherited nutritional marker were selected. For an explanation on the genotypes of the diploids, see text.

TABLE 20

Complementation tests on grc mutations

Diploid	Ratio $GSA^{sensitive}$:$GSA^{resistant}$ in 4-spore tetrads		
	# of Tetrads	2:2	1:3	0:4
3A x 15A	15	0	0	15
GSA^{r}-5 x 6C	12	3	8	1
Expected for a Dihybrid		1	4	1

DISCUSSION

We have isolated two mutants, GSA^r-4 and GSA^r-5 (both were derived from M18-23E) that show glucose insensitivity to two enzyme complexes of the respiratory chain. The enzymes NADH : cytochrome c reductase and cytochrome c oxidase show nearly derepressed values when the cells are grown on 5% glucose medium. The genetic experiments performed on these mutants reveal the participation of at least three loci in the glucose repression of mitochondrial biogenetic process. The three loci are GRC1, GRC2 and GRC3. The presence of grc1-1 and grc2 in GSA^r-4 and grc1-4 and grc3 in GSA^r-5 determines the glucose insensitive functioning of the respiratory chain. This is indicated by the rate of oxygen consumption of 5% glucose grown cells which is almost as high as fully derepressed 2% lactate grown cells. This derepression in turn allows for the growth on lactate in the presence of repressing levels of glucosamine.

Mutation at the GRC1 and GRC2 loci in GSA^r-4 and at GRC1 GRC3 loci in GSA^r-5 have been assayed for the enzymes of the respiratory chain and some other enzymes. These included δ-aminolevulinate dehydratase, citrate synthase, maltase, α-galactosidase and galactokinase. From our results discussed in chapter 3, it is seen that grc1-1 and grc2 in GSA^r-4 are involved in the derepression of NADH : cytochrome c reductase and cytochrome c oxidase. The loci, however, are not involved in glucose regulation of other respiratory enzymes

such as succinic : cytochrome c reductase, succinic dehydrogenase and NADH dehydrogenase. Similarly, the mutations grc1-4 and grc3 identified in GSA^r-5 are involved only in the derepression of NADH : cytochrome c reductase and cytochrome c oxidase and not for the other enzymes of the respiratory chain. Considering that GSA^r-4 and GSA^r-5 have been isolated independently from M18-23E and yet one gene GRC1 is common to both of them, it would imply that only a limited number of genes are operating in derepression of mitochondrial functions. All three grc mutations are nuclear genes.

The independent genetic control of the various complexes of the respiratory chain is interesting in light of the studies of the derepression of these enzymes (42, 55). Both Perlman & Mahler (42) and Kim and Beattie (55) found that the enzymes cytochrome c oxidase and NADH: cytochrome c reductase are regulated independently and also cytochrome c oxidase is the last enzyme to be derepressed during the derepression studies (55). The two mutations we have isolated in GSA^r-4 and GSA^r-5 may also be regulating these two enzyme complexes separately but since the effect of each gene is thought to be tightly linked to the other gene it may not be possible to study their expression in an environment devoid of the other gene. This hypothesis is strengthened by the observations that only presence of grc1 grc2 and grc1 grc3 in a spore results in a glucosamine resistant phenotype while grc2 grc3 combination exhibits glucose sensitivity. Thus, it has been postulated that grc1-1 and grc1-4 (wild type GRC1) are regulating one segment of the

respiratory chain and can interact with either grc2 or grc3 (both these loci are perhaps regulating another segment of the chain not controlled by the GRC1 locus) to produce glucosamine resistance and glucose insensitivity of the electron transport chain.

Kim and Beattie (55) have also found that succinic: cytochrome c reductase and NADH: cytochrome c reductase are regulated independently of each other. The response of these two enzyme complexes to protein synthesis inhibitors such as chloramphenicol and cycloheximide was found to be different suggesting that only NADH: cytochrome c reductase contained products of the mitochondrial protein synthesizing system. Also, the derepression studies on 5% glucose indicated that the succinic: cytochrome c reductase activity increases as soon as glucose is exhausted from the medium while a considerable lag occurs before the derepression of NADH: cytochrome c reductase begins. This differential regulation during glucose derepression was attributed to factors other than the primary dehydrogenases, NADH dehydrogenase and succinic dehydrogenase. Our studies support the hypothesis that succinic: cytochrome c reductase and NADH: cytochrome c reductase are regulated differently. The GRC loci regulate only complex I + III (NADH: cytochrome c reductase) and complex IV (cytochrome c oxidase) and appear to have no effect on complex II or complex II +III (succinic: cytochrome c reductase).

A number of mutations which affect glucose repression have been isolated in yeast. Montenecourt et. al (151) isolated a yeast mutant which exhibited less sensitivity of invertase and maltase to glucose repression. However, no genetics was reported on this mutant. Schmart et. al (152) isolated a similar mutant, flaky, which is glucose resistant for the synthesis of maltase, invertase, gal-1-P uridyltransferase and succinic dehydrogenase. Interestingly, the level of cyclic AMP was found to be similar in both the flaky and wild type strains grown under various growth conditions.

Zimmermann and co-workers have reported isolation of a large number of glucose repression resistant mutants. Zimmermann and Eaton (85) isolated revertants of a mal2 non-fermenting mutation which are resistant to glucose repression and are allelic to MAL2. Zimmermann et. al (1977) (153) have also isolated glucose resistant mutations starting with a cat1 strain. This strain does not grow on glycerol, does not ferment maltose and the derepression of isocitrate lyase, malate dehydrogenase, fructose, 1-6, diphosphatase and maltase is prevented. The respiration rate and the repression of invertase was found to be normal. Mutant revertants were obtained from the cat1 strain and these mapped in two loci. These strains were altered in their derepression of the enzymes mentioned above. Interestingly, all the affected enzymes (except maltase) in cat1 are glucose inactivated enzymes (note though that maltose permease is also a glucose inactivated enzyme (52)). Therefore, it is possible that the cat1 mutation and the revertants obtained from it are altered in their regulation of glucose inactivation rather than glucose repression.

In a subsequent study, Zimmermann and Scheel (154) used two mutant alleles of the CAT1 gene to isolate 2-deoxyglucose resistant mutants on raffinose. Only the mutants with glucose resistant invertase should be able to grow. The mutants obtained from this group exhibited glucose insensitive functioning of maltase, invertase and malate dehydrogenase. These mutants mapped in three genes. The phenotypes of two of the mutants resulted from a defect in sugar uptake (Entian et. al (155)) while the third gene has not been characterized.

Rytka et. al (96) have isolated mutants that exhibit glucose insensitive functioning of catalase. Two genes cgr1 and cgr2 are proposed but they have not been well characterized. The enzymes of the respiratory chain and gluconeogenetic pathway are found to be unaffected by these mutations.

Ciriacy (1977, 1978) (156,157) has isolated mutants that show resistance to repression by glucose. Mutations at the loci ccr1, ccr2 and ccr3 do not allow for growth on glycerol and ethanol. The mutants resemble those of Zimmermann et. al (153) in that the derepression of isocitrate lyase, fructose, 1-6, diphosphatase, alcohol dehydrogenase II and cytoplasmic dehydrogenase is prevented (all these enzymes are glucose inactivated). The TCA cycle enzymes and succinic dehydrogenase are unaffected. Thus, these mutants may be altered in the regulation of glucose inactivation. In a more recent report by Ciriacy (157) another class of mutant, mapping in a single locus CCR80, have been identified. The mutants exhibited glucose insensitive synthesis of succinic dehydrogenase and NADH:

cytochrome c reductase. The enzymes of the gluconeogenic pathway were not significantly altered. Most importantly, glucose fermentation is strongly inhibited in CCR80 mutants although the enzymes of glycolysis are present in normal levels. This may reflect a defect in the sugar uptake system of the mutants. Thus the phenotype of glucose resistance may be a result of glucose uptake defect.

Clearly, when one critically reviews these mutants from the previous studies it becomes clear that true 'glucose repression' resistant mutants may not exist. The class of mutants isolated here, GSA^r-4 and GSA^r-5, are phenotypically different from the mutants isolated above and most importantly these mutants grow normally on glucose. Therefore, the glucose insensitivity of the respiratory enzyme complexes is due to an actual resistance to glucose rather than due to some defect in sugar uptake.

At this point, the mechanism of action of the grc mutations can only be briefly speculated. It is possible that GRC1 is a major gene that requires the participation of another minor gene GRC2 or GRC3 to derepress NADH: cytochrome c reductase and cytochrome c oxidase. Alternatively, it can be suggested that GRC1 regulates half of the respiratory chain (i.e. either the NADH: cytochrome c reductase half or the cytochrome c oxidase half) and GRC2 and GRC3 regulate the half not derepressed by the GRC1 gene, and, since the effect of each gene is so much dependent on the other gene, their expression alone cannot be measured through the enzyme assays. The presence of both the

genes is required to study their affect on the respiratory chain.

The appearance of enzyme activities tested here can be regulated on many levels from transcription of the structural genes to the insertion of the final polypeptides into the mitochondrial membrane. It is already known that the enzymes maltase and δ-aminolevulinate dehydratase are regulated at the level of transcription during glucose repression (77,61). Cytochrome c is regulated both transcriptionally and post-transcriptionally by glucose (56, 57). The amount of CYC1 mRNA was measured by hybridization to cloned CYC1 gene, both before and after the addition of glucose, and it was found that the rate of CYC1 mRNA synthesis was reduced upon addition of glucose. Also, the existing CYC1 mRNA was rendered untranslatable by an endonucleolytic cleavage. The enzyme regulation can also take place post-translationally as in the case of invertase (128).

Among the other regulatory mechanisms to be considered are the following. The regulation might also be achieved by controlling the synthesis of a key subunit of cytochrome c oxidase. It is already known that the subunit III of cytochrome c oxidase (coded for by the OXI II region of mitochondrial DNA) is more sensitive to glucose repression that any of the other subunits. Hence, we can achieve glucose insensitivity of cytochrome c oxidase by reducing glucose insensitivity of the synthesis of subunit III via the participation of a nuclear gene. Therefore, if one were to study the response to glucose of different structural genes coding for these

7 subunits of cytochrome c oxidase, one would find the subunit III structural gene to exhibit less sensitivity to glucose. A similar mechanism of reduced sensitivity of a key polypeptide of NADH: cytochrome c reductase complex can be proposed to function in the glucose insensitivity of this enzyme.

It is interesting to note that the only two enzyme complexes that have achieved glucose insensitive synthesis in GSA^r-4 and GSA^r-5 are the cytochrome c oxidase and the NADH: cytochrome c reductase. Both these enzymes are assembled from the mitochondrial and cytoplasmic translation systems. The other enzyme complex of such dual origin is mitochondrial ATPase which has not been looked at in our work. Thus glucose insensitivity can also be achieved during the processing and maturation of the cytoplasmically made subunits of these two enzyme complexes. All these hypothesis will have to be tested in order to determine which one is operating in GSA^r-4 and GSA^r-5.

The regulatory effect of grc mutations can also occur at the level of insertion of prosethetic groups.

Recently, in our laboratory another class of glucosamine resistant mutants have been isolated on maltose. These grm mutations are insensitive to glucose repression for a wide variety of glucose regulated enzymes. It is possible that such grm mutations are related to the primary metabolic event(s) induced in the cell by glucose (such as the level of cyclic AMP, as in E. coli). However, grc mutations isolated in

this study cannot be related to such functions due to their specificity of action on only two enzyme complexes of the respiratory chain. Work is currently under way in our laboratory to isolate some more grc mutations in hope of identifying all nuclear genes (and mitochondrial genes, if existing) involved in the derepression of respiratory enzymes of the electron transport chain.

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