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Glycolytic Enzymes and Intermediates in Carbon Catabolite Repression Mutants of *Saccharomyces cerevisiae*

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Summary. Glycolytic parameters were determined in recessive yeast mutants with partial defects in carbon catabolite repression. Specific activities of pyruvate kinase and pyruvate decarboxylase in glucose grown cells of all mutant and wild type strains were 4–5 times higher than in ethanol grown cells. Mutants of gene HEX1 had a reduced hexose phosphorylating activity on all media whereas those of gene HEX2 had elevated levels but only in glucose grown cells. Mutants of gene CAT80 were normal in this respect. All other glycolytic enzymes were normal in all mutants. This was also true for glycolytic intermediates. Only hex1-mutants showed a reduced fermentation of repressing sugars. The three genes appear to be involved in catabolite repression of several but not of all repressible enzymes. Even though all three types of mutants show a limited overlap in their effects on certain enzymes, they still are distinctly different in their action spectra. Carbon catabolite repression apparently does not depend on the sole accumulation of glycolytic intermediates. The activity of the products of the three genes HEX1, HEX2 and CAT80 are required directly or indirectly for triggering carbon catabolite repression. Even a small segment of carbon catabolite repression is controlled by several genes with regulatory functions indicating that the entire regulatory circuit is highly complex.

Introduction

The enzymatic machinery of carbon catabolism in the yeast *Saccharomyces cerevisiae* and in many other microorganisms varies drastically with the type of carbon source in the medium. The presence of carbon sources which can be degraded via glycolysis prevents or reduces the formation of enzymes in the Krebs cycle, glyoxylate shunt, gluconeogenesis and the respiratory chain (see: Polakis and Bartley, 1965; van Wijk et al., 1969; Perlman and Mahler, 1974). Moreover, the formation of enzymes involved in the degradation of disaccharides like maltose and sucrose is also repressed by hexoses (van Wijk et al., 1969). This is now called carbon catabolite repression a term first used to describe similar conditions in bacteria (Magasanik, 1961). The analysis of the genetics of a regulatory system with such a wide action spectrum is a true challenge. Montenecourt et al. (1973) were the first to report on a mutant affecting carbon catabolite repression of several enzymes, but genetic analysis was not possible in this case. Schamhart, et al. (1975) isolated and genetically characterized a recessive mutant that affected the regulation of several enzymes. More recently, Ciriacy (1978) described and analysed dominant mutants of a gene called *CCR80* which eliminated carbon catabolite repression of almost all repressible enzymes. In contrast to this, Zimmermann and Scheel (1977) identified recessive mutants in three different genes which affected carbon catabolite repression of a limited spectrum of enzymes. Even though the regulatory defects in those mutants were strong, it was not clear whether the primary effects were due to reduced glycolysis or defects in genuine regulatory genes. In this article we report on a more thorough analysis of the three types of mutants.

Materials and Methods

Yeast Strains. All strains were derived through a series of crosses from strains orignating from the Department of Genetics of the University of Washington, Seattle, Wn.

The following mutant/wild type pairs were used:

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cat2.3-2A/18 *a his4 MAL3 SUC3 MAL2-8^c hex1-18* a direct mutant derivative of cat2.3-2A wild type.

hex1-38: SMC-1B/3000.38 *a his4 MAL3 SUC3 MAL2-8^c hex1-38* a direct mutant derivative of SMC-1B wild type.

hex2-3: SMC-1B/3 a direct mutant derivative of SMC-1B.

cat80-24: T.24-3B *alpha his4 MAL3 SUC3 MAL2-8^c cat80-24* is a sister sporling of strain T.24-2B derived from the same diploid as T.24-3B with *alpha MAL3 SUC3 MAL2-8^c*.

Gene Symbols. a and *alpha* are mating type alleles; *his4* causes a recessive histidine requirement. *MAL3* and *SUC3* are dominant and tightly linked genes promoting formation of maltase and invertase respectively; *MAL2-8^c* is a dominant mutant allele of *MAL2* a presumably positive regulatory gene for maltase synthesis which causes a partly constitutive but still glucose repressible synthesis of maltase (Zimmermann and Eaton, 1974).

Media. The basic medium consisted of 1% yeast extract and 2% peptone supplemented with glucose or ethanol. All growth experiments were performed at 28° C.

Crude extracts were prepared after Ciriacy (1975) and centrifuged in a Sorvall RC-2B centrifuge in an SS34 rotor at 15,000 rpm for 15 min or – when respiratory enzymes had to be determined – in a table top centrifuge at 2,000 g for 10 min.

Protein was determined by the microbiuret method of Zahmenhoff (1957) using serum albumin as a standard.

Enzyme Determinations. Total α-glucosidases using p-nitrophenyl-α-D-glucopyranoside as a substrate (maltase E.C. 3.2.1.20 and isomaltase E.C. 3.2.1.10), invertase (E.C. 3.2.1.26), isocitrate lyase (E.C. 4.1.3.1), total malate dehydrogenase (E.C. 1.1.1.37), and fructose-1,6-bisphosphatase were assayed as previously described (Zimmermann et al., 1977); malate synthase (E.C. 4.1.3.2) after Dixon and Kornberg (1959); succinate dehydrogenase (E.C. 1.3.99.1) with cytochrome c as an electron acceptor (Arrigoni and Singer, 1962), NADH-dehydrogenase (E.C. 1.6.99.3) after Duncan and Mackler (1966); hexokinases (E.C. 2.7.1.2 and 2.7.1.4), phosphoglucose isomerase (E.C. 5.3.1.9), aldolase (E.C. 4.1.2.7), glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12), 3-phosphoglycerate kinase (E.C. 2.7.2.3), phosphoglucomutase (E.C. 2.7.5.3), enolase (E.C. 4.2.1.11) and pyruvate kinase were determined after Maitra and Lobo (1971); phosphofructokinase (E.C. 2.7.1.56) after Gancedo and Gancedo (1971) and pyruvate decarboxylase (E.C. 4.1.1.1) according to Polakis and Bartley (1965).

Determination of Metabolites. In the case of mutant strains with *hex1-18* determinations were performed as described by Entian et al. (1977). In the case of strains with mutant alleles *hex2-3* and *cat80-24* and their respective wild types, a slightly modified procedure was followed. Culture media were pipetted onto ice of −25° C in centrifuge tubes and centrifuged for 25 s at 5,000 rpm in a Christ Labofuge III. During this process, the ice melted and the supernatant had reached about 4° C. After pouring off the supernatant, the cells were resuspended in boiling ethanol. In the case of NADH and NADPH, a mixture of 1 volume of a 500 mM glycine buffer pH 9.6 and 9 volumes of ethanol was used. Only 1,3-diphosphoglycerate was too unstable to be determined in such extracts. Finally, all metabolites were determined after Bergmeyer (1970).

Fermentation and respiration rates were determined by standard Warburg manometry as described before (Entian et al., 1977) and expressed as nmoles CO_2 produced (corrected for respiratory CO_2-production) and O_2 consumed per min and mg cell protein. The protein concentration was estimated from a standard curve using dry weight determinations (Entian, 1979).

Results

The three types of mutants studied in the present communication were isolated on the basis of invertase formation that was no longer subject to carbon catabolite repression. Even though over 600 mutants were isolated on this basis using a medium with 2-deoxyglucose and raffinose, all mutants could be located in only three genes (Zimmermann and Scheel, 1977; Entian, 1979). One class studied in greater detail was assigned the symbol *hex1*, wild type *HEX1*, and was not only deficient in carbon catabolite repression of certain enzymes but also in carbon catabolite inactivation (Entian et al., 1977; Entian, 1977, 1979). Catabolite inactivation describes another type of regulatory system in carbon metabolism which is based on the inactivation of a number of glyoxylate shunt and gluconeogenic enzymes when glucose or fructose are added to yeast cultures growing on a non-carbohydrate

Table 1. Specific activities of repressible enzymes in mutants and wild types after growth on repressing glucose and non-repressing ethanol media

	PNPG		INV		MDH		SDH		NADH		MS		FBP		ICL	
	rep.	der.	rep.	der.	rep.	der.	rep.	der.	rep.	der.	rep.	der.	rep.	der.	rep.	der.
HEX1	1.8	86	16	813	169	2627	1.5	48	9.8	67	2.5	223	1.5	61	2.8	98
hex1-18	285	92	1,280	927	1,500	2,907	12.0	51	29.1	72	1.4	251	0.8	59	3.8	97
HEX2	1.4	89	28	917	253	2,317	1.2	58	12.0	76	1.2	247	1.8	67	2.5	83
hex2-3	124	87	2,118	1,023	1,304	2,528	30.0	60	42.0	78	1.8	271	2.1	64	2.7	97
CAT80	1.2	83	28	927	312	2,142	1.2	56	8.5	78	2.0	234	1.2	49	1.2	49
cat80-24	78	81	1,423	1,378	2,934	2,880	1.8	54	7.2	74	1.8	258	1.5	53	1.5	84

Specific activities in nmoles/min × mg protein. PNPG = alphaglucosidases; INV = invertase; MDH = total malate dehydrogenase; SDH = succinate dehydrogenase; NADH = NADH-dehydrogenase; MS = malat synthase; FBF = fructose-1,6-bisphosphatase; ICL = isocitrate lyase; rep. = grown on 2% glucose YEP; der. = grown on 3% ethanol YEP. *HEX1, HEX2* and *CAT80* are wildtype, *hex1-18, hex2-3* and *cat80-24* are mutant strains

Table 2. Specific activities of glycolytic enzymes in mutants and wild types after growth on glucose and ethanol media

	HEX1		*hex1-38*		*HEX2*		*hex2-3*		*CAT80*		*cat80-24*	
	glu.	eth.	glu.	eth.	glu.	eth.	glu.	eth.	glu.	eth.	glu.	eth.
glu-kinase	1,143	1,180	145	138	991	1,169	2,585	1,175	883	907	987	1,023
fru-kinase	1,287	1,527	283	274	1,442	1,726	3,524	1,681	1,231	1,482	1,183	1,296
PGI	794	708	692	765	1,154	872	1,053	767	638	627	681	591
PFK	405	324	381	307	383	295	335	283	338	301	347	283
aldolase	44	33	38	35	50	48	23	24	38	34	36	35
GLD	412	381	362	375	386	380	296	255	473	394	412	377
GK	1,528	1,297	1,473	1,312	1,643	1,553	1,307	1,387	1,371	1,384	1,351	1,573
PGM	887	821	858	802	832	801	746	811	812	789	776	812
enolase	2,227	1,984	2,103	2,007	1,919	1,983	1,429	1,445	1,857	1,692	1,917	1,829
PYK	2,033	470	2,124	521	1,998	443	1,943	423	1,992	412	2,102	527
PDC	1,111	274	1,223	299	1,125	203	1,119	218	992	183	974	205

Specific activities in nmoles/min × mg protein. Glu-kinase = glucose phosphorylation; fru-kinase = fructose phosphorylation; PGI = phosphoglucose isomerase; PFK = phosphofructokinase; GLD = glceraldehyde-3-phosphate dehydrogenase; GK = 3-phosphoglycerate kinase; PGM = phosphoglyceromutase; PYK = pyruvate kinase; PDC = pyruvate decarboxylase. Strain designations as in Table 1

Table 3. Fermentation and respiration rates of mutant and wild type strains on media with different carbon sources

	Fermentation of		Respiration		
	glucose	galactose	glucose	galactose	ethanol
HEX1	1,030	–	30	–	250
hex1-18	454	–	49	–	255
HEX2	1,700	800	30	500	520
hex2-3	1,500	500	380	720	590
CAT80	2,145	642	40	297	550
cat80-24	2,582	736	113	287	505

Cells were grown overnight on the same media in which fermentation and respiration was determined. All measurements in growth medium at 30° C. Fermentation = nmoles of fermentative CO_2 per min × mg protein; respiration = oxygen consumption in nmoles/min × mg protein. Sugar concentrations in the growth media 2%, for ethanol 3%. Final sugar concentrations in the Warburg vessels 1.6%

carbon source (Holzer, 1976). The other two types of mutants are now called *hex2* and *cat80*, and their respective wild type alleles *HEX2* and *CAT80*. *hex2*-mutants are strongly inhibited by maltose (Zimmermann and Scheel, 1977; Entian, 1979) whereas *cat80*-mutants show no particular abnormalities. We have now compared mutant alleles hex1-18, hex1-38 and hex2-3 to their wild type ancestors or in the case of cat80-24 to a wild type sibling segregating from a common diploid parent heterozygous for cat80-24.

The repression spectrum of the three mutant types is shown in Table 1. Repression of total α-glucosidase activities, invertase, and total malate dehydrogenase activities were affected in all three types. Mutants with *hex2-3* or *hex1-18* showed also reduced repression of succinate and NADH-dehydrogenase. However, malate synthase and isocytrate lyase in the glyoxylate shunt and fructose-1,6-bisphosphatase were found at fully repressed levels in all three classes of mutants. Glycolytic enzymes were determined in cells grown on ethanol and on glucose media (Table 2). The only difference between these two growth conditions was that pyruvate kinase and pyruvate decarboxylase were much lower in cells grown on ethanol. No differences between mutant and wild type were observed in the mutant strain with *cat80-24*. As reported previously (Zimmermann and Scheel, 1977), the *hex1*-mutation reduced the overall activity of hexose phosphorylation. In the case of *hex2*-strains, overall glucose phosphorylation was increased over wild type but only when grown on glucose.

Utilization of glucose was determined under fermentative conditions by Warburg manometry (Table 3). Unfortunately, fermentation rates were already different between the three wild type strains. However, a comparison between the three wild types and their mutant derivatives revealed a reduced fermentation rate only for the *hex1*-mutation as described before (Entian et al., 1977). Mutations *hex2-3* and *cat80-24* had no effects on the glycolytic flux.

Even though the determinations of glycolytic enzymes and fermentation rates were abnormal only in the case of mutation *hex1*, glycolytic intermediate concentrations were determined in all three mutant types. As can be seen in Fig. 1, there were no striking differences between mutants and their wild type parent strains.

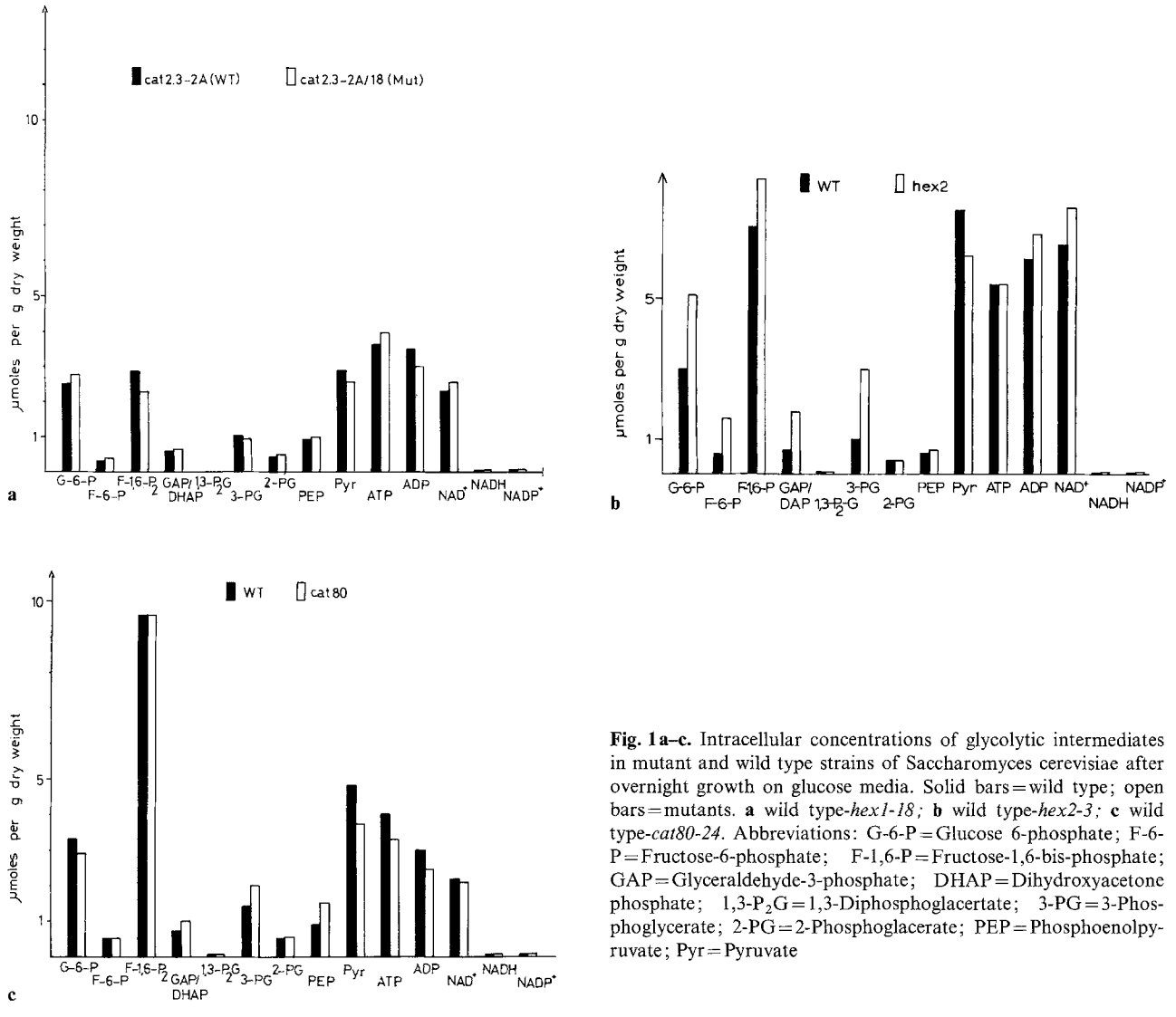

Fig. 1a–c. Intracellular concentrations of glycolytic intermediates in mutant and wild type strains of Saccharomyces cerevisiae after overnight growth on glucose media. Solid bars = wild type; open bars = mutants. **a** wild type-*hex1-18;* **b** wild type-*hex2-3;* **c** wild type-*cat80-24.* Abbreviations: G-6-P = Glucose 6-phosphate; F-6-P = Fructose-6-phosphate; F-1,6-P = Fructose-1,6-bis-phosphate; GAP = Glyceraldehyde-3-phosphate; DHAP = Dihydroxyacetone phosphate; 1,3-P_2G = 1,3-Diphosphoglacertate; 3-PG = 3-Phosphoglycerate; 2-PG = 2-Phosphoglacerate; PEP = Phosphoenolpyruvate; Pyr = Pyruvate

Discussion

Invertase synthesis resistant to carbon catabolite repression was the selected property in the isolation of the mutants described in this article. Altogether over 600 mutants isolates were analysed but all had to be assigned to one of the three genes *HEX1, HEX2* and *CAT80* (Zimmermann and Scheel, 1977; Entian, 1979). Significantly absent were mutants belonging to genes that specifically affected the regulation of invertase synthesis on one hand, and mutants with a very wide action spectrum affecting all functions subject to carbon catabolite repression. Such mutants were actually obtained by Ciriacy (1978) selecting for non-repressible respiratory activity. The primary effects of *hex1*- and *hex2*-mutants were observed in the system for hexose phosphorylation. Mutants *hex1* were analysed and found to be affected in a structural component of the hexose phosphorylation system (Entian, 1977) whereas *hex2*-mutants appeared to be somewhat affected in the regulation of synthesis of the components of this system (Entian, 1979). They differed most markedly in respect to catabolite inactivation which was reduced or abolished in *hex1*-mutants (Entian, 1977) but normal in *hex2*-mutants (Entian, 1979). The action spectrum of the *cat80*-mutant was limited to invertase, α-glucosidases and part of the malate dehydrogenase system. The effects of *hex1*- and *hex2*-mutants also covered respiration in general and succinate- and NADH-dehydrogenase.

There are at least three genes involved in the regulation of invertase synthesis, but they are not merely in one straight regulatory pathway, they seem to represent branching points. *HEX1* affects catabolite re-

pression and inactivation, *HEX2* affects repression alone but not inactivation whereas *CAT80* appears to have the smallest action spectrum.

None of the mutants affected repression of enzymes of the glyoxylate shunt and gluconeogenesis. Of course, it is difficult to make such a general statement because most of these enzymes are not only subject to repression but also to inactivation (Holzer, 1976). Only malate synthase is exempt from inactivation (Duntze et al., 1969), and this enzyme was always low under repressing conditions suggesting that repression was normal. In the case of *hex1*-mutants, catabolite inactivation was abolished but nevertheless, all the enzymes subject to inactivation were very low under repressing conditions. This suggests that probably none of the three genes is involved in the repression of gluconeogenic and glyoxylate shunt activities. Apparently, different functions subject to catabolite repression are under the control of more specific genes. It is not clear at present whether there is a "master regulatory gene". Gene *CCR80* of Ciriacy (1978) would be a candidate for such a function. However, the dominant non-repressible *CCR80*-mutants show reduced glycolytic activity and could therefore have a primary defect elsewhere but not necessarily in a regulatory gene proper.

A very obvious trigger for catabolite repression and also inactivation could be the increase of certain glycolytic intermediates in cells being transferred from a non-repressible ethanol to a repressing glucose medium. However, none of the three mutant classes showed any evident abnormalities in glycolytic metabolite concentration. If such metabolites are the triggers for catabolite repression, they can only do so in combination with products of – at least – genes *HEX1, HEX2* and *CAT80*. Mutation in any one of those genes blocks repression of certain enzymes. The question is then whether the three genes are specific regulatory of bifunctional genes i.e. do their products exert catalytic as well as regulatory functions? In the case of *HEX1*, the evidence is in favor of a bifunctional nature.

The genes described in this article were recognized on the basis of mutants with a defect in repression. Another approach is to identify genes on the basis of a blocked derepression. Such an approach has yielded five more genes: *CAT1, CAT2* (Zimmermann et al., 1977) and *CCR1, CCR2* and *CCR3* (Ciriacy, 1977). Mutant alleles of those genes affected mainly derepression of enzymes of the glyoxylate shunt and gluconeogenesis. Even though it is impossible at present to propose a full model of the genetic components of the catabolite repression system, it is obvious that it must be highly differentiated and certainly more complex than one based on the variation of a simple low molecular weight compound.

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