A GLYCINE TO SERINE SUBSTITUTION IDENTIFIES THE CYP 3 LOCUS AS THE STRUCTURAL GENE OF ISO 2 CYTOCHROME C IN *SACCHAROMYCES CEREVISIAE*

Eric Petrochilo and Jacqueline Verdière

Centre de Génétique Moléculaire du C.N.R.S., 91190 Gif-sur-Yvette, France

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Summary. The CYP 3 locus which was until lately considered as a regulatory gene for the synthesis of iso-2 cytochrome c in *Saccharomyces cerevisiae* has now been identified as the structural gene of iso-2 cytochrome c by the analysis of a mutant protein in which it was found that the invariant glycine at position 6 (horse cytochrome c nomenclature) is replaced by a serine residue. This replacement can be explained by a G-C to A-T transition, induced by the mutagenic agent ethyl methyl sulfonate used to obtain the mutant strain. Moreover a revertant was obtained in which the original mutation was conserved and the activity of the protein was apparently restored by an unidentified modification of residue 52, an invariant asparagine. In view of these results, the regulatory mutations previously obtained (1) are interpreted as "up promoter" mutations.

INTRODUCTION

As already described (2) *Saccharomyces cerevisiae* synthesises two species of cytochrome c which are simultaneously present in the cell although in very unequal amounts. Iso 1-cytochrome c the product of the CYC 1 locus (3) is the major species accounting for up to 95 % of the total cytochrome c. Although the sequence of iso-2-cytochrome c, the minor species differs by 20 residues from that of iso-1, the two cytochromes c appear to be isofunctionnal by all criteria so far applied. Furthermore a number of regulatory loci have been described : some inhibit the synthesis of both cytochromes (3), others enhance the synthesis of iso-2-cytochrome c while inhibiting that of iso-1-cytochrome c (1); finally regulatory mutants at the CYP 3 locus enhance specifically the synthesis of iso-2-cytochrome c (1) and do not affect that of iso-1.

In a previous paper (4) it was suggested that this last locus might well be that of the iso-2-cytochrome c structural gene and a mutant at this very locus synthesising a non functionnal iso-2-cytochrome c was described. We now present full evidence that CYP 3 is indeed the structural gene of iso-2-cytochrome c by showing that the mutant protein has an altered primary structure, the invariant glycine at position 6 (horse cytochrome c nomenclature) being replaced by a serine residue.

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MATERIALS.

The following products were purchased respectively :
Amberlite CG 50611 from Serva,
Dithiothreitol from Sigma.
2-mercaptoethanol from Merck.
Carboxymethylcellulose CM 52 from Whatman.
Thin layer Silica gel plates 0.25 mm thickness whith fluorescence indicator from Merck.

METHODS.

Isolation of cytochrome c.

Extractions and the first steps of purification were carried out according to the procedure described in (2). The cytochrome was eluted from the amberlite ion exchanger by a NaCl gradient ranging from 0.15 to 0.3 M in 0.1 M K/Na_2 phosphate buffer (Sorensen buffer) pH 7 containing 2.10^{-3} M dithiothreitol.

Fractions containing modified or wild type iso-2-cytochrome c were collected and part of the contaminants were discarded by precipitation with ammonium sulfate to 70 % saturation.

After centrifugation the supernatant was extensively dialysed against 2.10^{-2} M pH 7 Sorensen buffer containing 10^{-2} M mercaptoethanol. The dialysed solution was mixed with a small amount of CM 52 Carboxymethyl cellulose previously equilibrated whith the same buffer. In such conditions more than 90 % of the cytochrome c was adsorbed on the resin within a few minutes.

The cytochrome c containing resin was then layered on the top of a column of CM cellulose (50 cm long, 2 cm in a diameter) and eluted with a 24 mM pyrophosphate/HCl pH 8.5 buffer containing 2.10^{-3} M dithiothreitol. The cytochrome c containing fractions were gathered and extensively dialysed against 10 % acetic acid and stored at - 20°C.

Strains :

Wild type : DP9-23A, α his1-1, cyc1-1 cyp 1-18 CYP3
Mutant strain : VP 94-4A, α his1-1, CYC 1 cyp 1-18, cyp 3-1
Revertant strain : JA 1/R3 α his 1-1 cyc 1-1, cyp 1-18, cyp 3-1/R3.

The phenotype and obtainment of the mutant strain have already been described (4). The revertant was obtained by selection for growth on glycerol after U.V. irradiation of the original mutant JA 1 : α, his, cyc 1-1, cyp 1-18, cyp 3-1.

Characterisation of the proteins :

The purity of the preparations was ascertained by two independant criteria :

1 - Dodecylsulfate polyacrylamide gel electrophoresis was carried out as described in (5). Electrophoresis of wild type and mutant preparations produced a single band. Coelectrophoresis of mixture of the two proteins also produced a single band. This indicated that each preparation was pure, and furthermore that the molecular weight of the two proteins was identical.

2 - The N terminal amino acid was determined on 5 nmoles of the protein by the dansylation procedure described in (6). By far the major spot observed corresponded to Alanine, no significant contaminants being observed in either wild type or mutant protein preparations.

Tryptic hydrolysis was performed as described in (7) and peptide map analysis (8) was carried out by applying on silica gel plates samples of either 8 nmoles of tryptic hydrolysates dissolved in 1 µliter electrophoresis buffer for analytical peptide maps, or 40 nmoles of the sample in 5 µliters, buffer for preparative peptide maps.

Electrophoresis was performed at 300 volts for 135 min in a pyridine-acetate buffer pH 4.7 or 6.4 depending on the experiment.

Ascending chromatography was run in a pyridine-butanol-acetate-water

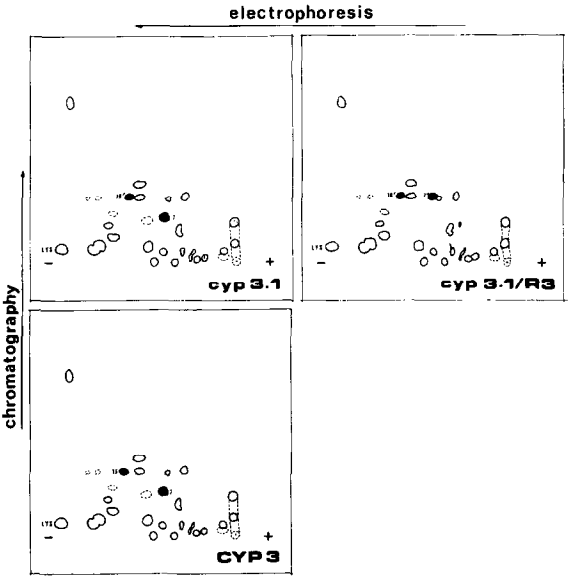

Figure 1. Peptide maps of the tryptic digests of the wild type (CYP 3 genotype), mutant (cyp 3-1 genotype) and revertant (cyp 3-1/R3 genotype) proteins. Experimental details are as described in Material and Methods. - points to the direction of the cathod; + to the direction of the anod. The numbers 7 and 16 indicate those peptides (composition in Table I) that were found to be modified in the mutant and revertant proteins. In the modified form they are referred to as 7' and 16' respectively.

(64-80-16-42) mixture at 18°C. The plates were dried at 80°C for 60 min and the peptides were retrieved, after detection either by ninhydrin collidine staining or by U.V. fluorescence, by scraping the silica gel from the plates and collecting it into one ml 5.7 N HCl.

Hydrolysis was carried out for 24 hours at 110°C in evacuated sealed tubes in the presence of 0.5 % 2-Mercaptoethanol.

Amino acid analysis were performed on a Beckman 120 C analyser using the two columns system ; the recorder was equiped with a 4 to 5 millivolts range card allowing good precision from 3 to 5 nmoles upwards. No distinction was made between dicarboxylic acids and their amides.

Nomenclature : Throughout this paper all amino acid residues are refered to by the positional number of their corresponding amino acid in the sequence of horse cytochrome c and not by their position in the iso-2-cytochrome c amino acid chain.

RESULTS

A. PEPTIDE MAPPING

An outline of the peptide maps obtained for each of the three proteins wild type, mutant, and revertant is shown in figure 1.

A single difference is detectable between the mutant and the wild type peptide maps. The difference is located at the level of peptide 16 and affects slightly the electrophoretic behaviour of the peptide.

The revertant has the same modification as the mutant as far as peptide 16 is concerned ; however it appears that peptide 7 is lacking in the revertant and replaced by a peptide 7' having the same electrophoretic mobility but a different chromatographic behaviour.

B. AMINO ACID COMPOSITION OF PEPTIDES 16, 7 AND 7'.

Table 1 shows the amino acid composition of peptide 16 for the three types of proteins. According to the published sequence of iso-2-cytochrome c (9), the composition of the wild type peptide 16 identifies it unambiguously as the tryptic fragment extending from position 6 to position 11 (horse cytochrome c nomenclature). The mutant and the revertant peptides show identical compositions and differ from the wild type peptide by the absence of a glycine and the presence of an extra serine residue. We conclude that the glycine residue at position 6 in the wild type has been replaced by a serine in the mutant.

Also shown in table 1 are the amino acid compositions of peptide 7 of the mutant protein and its corresponding peptide 7' in the revertant. The composition of peptide 7 is exactly that expected for the tryptic peptide extending from position 45 to position 55 according to the wild type sequence. The composition of peptide 7' differs from the expected and observed composition only by the absence of an aspartyl residue. Other minor differences can be accounted for as explained in the legend to table 1. Since there are three aspartyl residues in this peptide the amino acid composition alone is insufficient to decide which one is affected. However the identical behaviour of the two peptides (7 and 7') during electrophoresis, means that they must have the same overall electric charge, and since there is no additional negatively charged residue in peptide 7' it precludes that the affected residue be an aspartic acid. As it happens peptide 7 contains two aspartic acids and a single asparagine at position 52 (horse cytochrome c nomenclature). The amide group of asparagine is practically uncharged so that the deletion of this residue or its replacement by a neutral amino acid would not be expected to change the electrophoretic behaviour of the peptide ; it follows that the mutation that restores the activity of the revertant protein must have occured at position 52.

To our knowledge this is the first instance that an inactive mutant form of cytochrome c has been amenable to purification ; the behaviour of this cytochrome c in vitro might lead to some insight of electron transfer phenomena, all the more so, since a revertant is available whose function is restored by a further modification of the protein.

Table I. Amino acid composition of the modified tryptic peptides eluted from silica gel plates of the wild type (CYP 3 genotype), mutant (cyp 3-1 genotype) and revertant (cyp 3-1/R3 genotype) proteins.

The contaminants which are observed in relatively high amount in peptide 7' can be explained by its immediate proximity on the peptide maps (fig.1) to the tryptic peptide extending from position 28 to position 38 the amino acid composition of which explains more or less the nature of the contaminants.

Peptide 16 6-11	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe
Wild type	1	-	-	-	0.96	0.1	-	-	1.02	1.05	-	-	-	1	-	0.97
Mutant	1	-	-	-	1	0.81	-	-	0.16	0.94	-	-	-	1	-	0.98
Revertant	1	-	-	-	1	0.94	-	-	0.2	1.04	-	-	-	0.98	-	0.96
Peptide 7 45-55	**Lys**	**His**	**Arg**	**Asp**	**Thr**	**Ser**	**Glu**	**Pro**	**Gly**	**Ala**	**Val**	**Met**	**Ile**	**Leu**	**Tyr**	**Phe**
Mutant	1	-	-	2.99	1	1.04	-	-	0.82	1.08	-	-	1.01	-	1.89	-
Revertant	1	-	-	1.97	1	0.8	-	-	1.29	0.88	0.24	0.14	1.18	0.42	1.76	0.33
Theoretical	1	-	-	3	1	1	-	-	1	1	-	-	1	-	2	-

Table II. Partial sequence of iso-2-cytochrome c of the wild type protein (CYP 3 genotype) and presumptive sequences of the original inactive mutant (cyp 3-1 genotype) and of the revertant functionnal proteins (cyp 3-1/R3 genotype). The mutated residues are underlined.

Peptide 16						
Allele		6			10	
CYP 3	Gly	-Ala-Thr-Leu-		Phe	-Lys	
cyp 3-1	Ser	-Ala-Thr-Leu-		Phe	-Lys	
cyp 3-1/R3	Ser	-Ala-Thr-Leu-		Phe	-Lys	

Peptide 7									
Allele	45			50				55	
CYP3	Gly-Tyr-Ser-Tyr-Thr-Asp-Ala-	Asn	-Ile-Asp-Lys						
cyp 3-1	Gly-Tyr-Ser-Tyr-Thr-Asp-Ala-	Asn	-Ile-Asp-Lys						
cyp3-1/R3	Gly-Tyr-Ser-Tyr-Thr-Asp-Ala-	Δ ?	-Ile-Asp-Lys						

DISCUSSION

A modified inactive cytochrome c was purified and analysed by tryptic digest finger prints on thin layer silica gel plates. A single difference was observed between the digest of the wild type and that of the mutated protein. This difference concerned the tryptic peptide extending from position 6 to position 11. In this peptide glycine number 6 was replaced by a serine. This replacement is compatible with a transition of a GC to an AT base pair in the glycine codon, providing that glycine in position 6 be coded by GGU or GGC. Sherman (10) has shown that the codon for the same position in iso-1-cytochrome c is indeed GGU. It seems therefore that this mutation induced by E.M.S. is a straightforward transition.

Throughout all the known cytochrome c sequences, 12 residues have remained totally invariant. Glycine in position 6 is one of them ; it is not surprising therefore to find that the substitution of this invariant residue by a serine leads to a molecule which has no activity in vivo.

The exact nature of the modification that restores activity to the revertant protein is not clear ; taken at their face value, our data suggest that asparagine at position 52 might be deleted or possibly replaced by an unidentified residue. That the alteration in the revertant affects position 52 is however indisputable, and this is worthy of some comments :

- Asparagine at position 52 is invariant in all known eukaryotic cytochromes c, so that the replacement by a serine residue of the invariant glycyne at position 6 can be compensated by altering another invariant residue

at position 52. This is a puzzling result since it implies that functionnal alternatives exist for even the most conserved residues.

- Position 52 is on the other side of the heme plane and roughly diametrically opposite to position 6, taking the heme iron atom as the center of the molecule, as can be seen on tridimensionnal models of cytochrome c ; this seems an unlikely location if the role of the secondary mutation were simply to compensate for a steeric hindrance introduced by the initial glycine to serine substitution (11).

Whatever the case, we feel that this mutant and its revertant offer an ideal system for studying the electron transfer process in vitro.

We have obtained fifteen mutants at the CYP 3 locus, some of them have various amounts of cytochrome c, while in others we have failed to detect the slightest trace of cytochrome c. **The obtainment** of these mutants and the extensive analysis of cyp 3-1 establishes once and for all that CYP 3 is the structural gene of iso-2 cytochrome c and proves that the total absence of cytochrome c is not lethal to the cell.

The CYP 3 locus was previously described as a regulatory locus and characterised by two dominant mutations enhancing specifically iso-2-cytochrome c synthesis : cyp 3-4 and cyp 3-15 (1).

These two mutations are in fact cis dominant (unpublished results) so that the contradiction between the two types of mutations is more apparent than real : mutations in the structural gene may result in the absence of cytochrome c, while mutations in the region adjacent and preceding the structural gene may have regulatory effects. This type of "up-promoter" mutation has by now been widely recognised in eukaryotic systems such as Yeast,Aspergillus and Drosophila to mention but a few (12, 13, 14,15,16) ; the cyp 3-15 and cyp 3-4 alleles were amongst the very first such mutations obtained, but were not recognised as such, because structural gene mutants were not available at the time. By finally obtaining structural gene mutations, we feel confident that the regulation of this system can now be studied fruitfully.

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