INDUCED CONVERSION OF A PROTEIN PRECURSOR INTO CYTOCHROME C PEROXIDASE DURING ADAPTATION OF YEAST TO OXYGEN

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Received June 7, 1968

Oxygen-inducible hemoproteins of yeast have been classified according to their structural integration into mitochondria : (i) The first group comprises the particulate electron carriers firmly bound to the mitochondrial structures (cyt.aa_3,b); their synthesis is, as a rule, inhibited by the agents which depress the biogenesis of mitochondria, chloramphenicol for instance (Huang et al, 1965). Their synthesis, futhermore, requires the integrity of the cytoplasmic information carried by the mitochondrial DNA (Mounoulou, 1967; Fukuhara, 1967). (ii) The second class includes cytochrome c and cytochrome c peroxidase which are easily lost from damaged mitochondria, and catalase operationally described as a soluble enzyme of the cytoplasm.

Oxygen-induction of cytochrome c peroxidase (abbreviated CcP) has been a subject of controversy; admitted by some (Chantrenne, 1955; Sels, 1960, 1962; Sels and Cocriamont, 1967) the inducibility of the enzyme was denied by others (Lindenmayer and Smith, 1964).

The antinomy observed between the mechanisms of synthesis of the individual iso-cytochromes c in yeast introduced a new criterion of distinction between hemoproteins. On the one hand, most of these hemoproteins are synthesized de novo from free amino acids during adaptation to oxygen. On the other hand, iso-2-cytochrome c is made from a macromolecular precursor requiring only the inclusion, under aerobic conditions, of heme or a precursor of the prosthetic group (Sels et al, 1965; Fukuhara and Sels, 1966; Fukuhara, 1966). The last type of induction mechanism, which we will refer to as "induced conversion", applies also to the formation of CcP when anaerobic yeast adapts to oxygen.

Characteristic properties of the induced conversion leading to the formation of CcP holoenzyme were found to be the following : 1) high sensitivity to traces of oxygen 2) rapidity 3) resistance to the action of cycloheximide (actidione), a powerful inhibitor of the de novo synthesis

of proteins in yeast (Fukuhara,1965,1966). These criteria are similar to those established for iso-2-cytochrome c (Sels et al,1965;Fukuhara,1966).

The fact that heme and the native apocytochrome c peroxidase (abbreviated apoCcP) preexisting in the anaerobic (non induced) yeast recombine directly in cell-free extracts,demonstrates unequivocally that the apoenzyme alone is constitutive.

Studies to discriminate between the possible coexistence in the same yeast cell of two distinct molecular specie of CcP or the coexistence of different structural states of a unique enzyme are in progress;this problem will be discussed elsewhere.

Results -

The autolysis technique originally devised by Abrams et al (1942) to extract CcP from yeast has also been used to demonstrate the induction of CcP during adaptation of yeast to oxygen (Chantrenne,1955;Sels,1960). We found however that this extraction procedure was unsuitable for studying the kinetics of the induction process. In effect,autolytic digestion of the yeast cells destroyed the CcP in proportions which varied mainly in function of the concentration of endogenous (also oxygen-induced) cytochrome c in the yeast sample analyzed (Sels,1962);this result was interpreted at first as a stabilization of the extracted enzyme by his substrate. For this reason we preferred mechanical disruption of the yeast cells in the cold (0°) using a French press. Previous to disruption the yeast pellet was suspended in an equal volume of Sörensen phosphate buffer 0.05M-pH 6.0. In the resulting crude extracts (i.e. the supernatant from two centrifugations at 3,000xg for 5 min.,referred to as "total extract",and in the supernatant from the centrifugation of the latter at 100,000xg for 60 min.,referred to as "S fraction") no appreciable decrease of CcP activity occured at 0° during the first 6 hours (even after 24 hours the loss of activity was generally negligible).

By the above mechanical disruption procedure CcP is released from the damaged mitochondria;the enzyme,which is solubilized,is recovered in the S fraction (90 to 100% with aerobic cells). CcP activity was assayed spectrophotometrically (using a Cary 15 or a Beckman DU monochromator-Gilford 2000 recorder combination) in Sörensen phosphate buffer 0.05M - pH 6.0 at 23°. The initial rate of the oxidation of 25 μM ferrocytochrome c (horse heart cytochrome c:Sigma Type VI),catalyzed by CcP,was measured using 200μM H_2O_2 as the oxidizing agent. The enzyme activities were compared in terms of millimoles of ferrocytochrome c oxidized/sec./mg protein in the total extract.

A factor of considerable importance, which may be a source of discrepancies, was the quality of anaerobiosis since we found that trace amounts of molecular oxygen were sufficient to maintain aerobic traits in the cultures, specifically as regards iso-2-cytochrome c and CcP production. Indeed, we observed that reducing the concentration of oxygen in the gas phase untill pO_2=1/500 did not depress significatively the yield of these two hemoproteins. On the other hand, when anaerobic culture conditions of the type described by Chantrenne were used (Chantrenne and Courtois, 1954; Chantrenne, 1955), the cells, when harvested at 0°, were already partially induced. In these conditions, when the exhaustion of molecular oxygen in the liquid phase is slow, the concentration of CcP in stationary phase cells already reached one third of the aerobic level, although the particulate hemoproteins were absent. For anaerobic growth a Yeast Extract-Glucose medium, enriched with wheat germ oil, was used (medium R according to Sels et al, 1965). Strictly anaerobic conditions were obtained by replacing air by highly purified nitrogen.

Table I - Yield of cyt.c peroxidase as a function of the physiological state of yeast.

Strain	Physiological state of yeast	Cyt.c peroxidase activity millimoles ferrocyt.c oxid./sec./mg protein
D261	anaerobic stat.phase	0.27 ± 0.11 (11)*
	aerobic stat.phase	7.1 ± 1.4 (4)*
	aerobic growth with chloramphenicol 4mg/ml	5.2 ± 1.1
YFA	anaerobic stat.phase	0.1
	aerobic stat.phase	4.5

* number of experiments

"Grande" ρ^+ strain D261 and "petite" ρ^- strain YFA (a vegetative mutant) were grown aerobically in a 1% Yeast Extract-1% Peptone-5% Glucose medium or under strict anaerobic conditions in medium R (see text). Stationary phase cells were harvested, washed and disrupted at 0°. Cyt.c peroxidase activity, associated with the S fraction, was assayed spectrophotometrically.

Table I shows that no appreciable CcP activity is detected when anaerobiosis is strictly controlled during growth and the cells are harvested at 0°. This is true for both a "grande" strain and a vegetative mutant. The phenotypic expression of the cytoplasmic factor (ρ^+) is not a prerequisite for the synthesis of CcP since the aerobic yield of enzyme is only slightly decreased (25% at a maximum) when the "grande" strain D261, grown aerobically in the presence of chloramphenicol 4mg/ml, acquires the

"petite" phenotype (cyt.aa_3 activity is completely abolished).

The "induced conversion" type of mechanism for the formation of CcP during adaptation of anaerobic yeast to oxygen was demonstrated by the use of cycloheximide. When the antibiotic was added under strict anaerobiosis, cycloheximide, dissolved in pure ethanol, was stored in a lateral appendix mounted on the culture flask. The drug was poured into the growth medium (final concentration 25µg/ml) when the cells had reached stationary phase, 15 min. before opening the flask (i.e. before contact of yeast with oxygen).

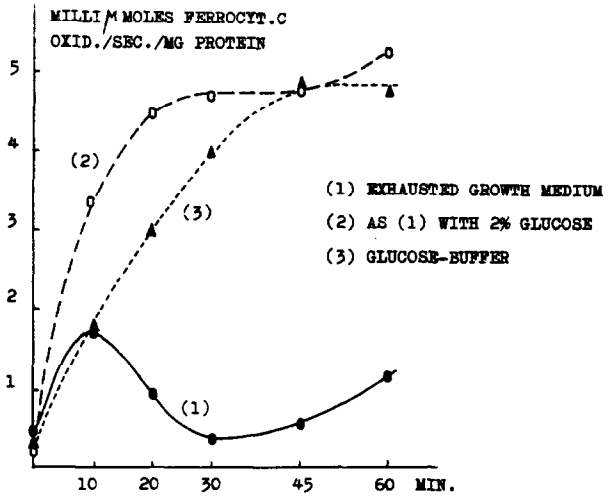

Fig.1 - Time course of cyt.c peroxidase production by cycloheximide-treated yeast cells.

Anaerobic stationary phase cells (D261), intoxicated by cycloheximide (25µg/ml) under strict anaerobiosis (see text), were aerated immediately at 25° in the exhausted growth medium (medium R) which was supplemented or not with 2% glucose. Previous to aeration another sample of the same yeast was harvested and washed at 0°; aeration started at 25° after the yeast cells were resuspended in 5% glucose-succinic acid/K succinate buffer (0.05M-pH 4.2) supplemented again with cycloheximide (25µg/ml). The cyt.c peroxidase activity, associated with the S fraction, was determined after various times of aeration (see text).

Figure 1 shows that the anaerobic, cycloheximide-intoxicated, yeast cells form CcP at high and maximum rate as soon as the aeration starts at 25°. When energy-supplemented media are used, the yield of enzyme is scarcely lower than after aerobic growth or adaptation in the absence of cycloheximide. When the aeration occurs in the exhausted growth medium (no energy) the initial burst of CcP production is followed by a decrease of the amount of extractable enzyme; this is indicative of *in situ* degradation of the holoenzyme.

The main conclusion is : CcP derives from an anaerobic protein precursor;the completion of the active holoenzyme,which requires only the inclusion of the prosthetic group in the preexisting apoprotein,occurs with an exceptionally high velocity. For this reasons we suggest that the "anaerobic" CcP described for the LK 2 G 12 strain (Lindenmayer and Smith, 1964) might actually be induced at a stage following the harvesting of the anaerobic yeast.

Apocytochrome c peroxidase obtained by chemical cleavage of the heme-protein link has been shown to recombine spontaneously with heme (Yonetani,1967). We therefore looked for a comparable activation mechanism in cell-free extracts of anaerobically grown yeast cells.

In crude total extracts,the recombination was temperature-dependent (0° to 25°) and required (i) 1 to 10µM heme (ii) 1.5 to 15µM cytochrome c.

Table II - Recombination of the native apocytochrome c peroxidase with heme in cell-free extracts of anaerobic yeast.

Conditions of incubation			Cyt.c peroxidase activity milliµmoles ferrocyt.c oxid./sec./mg protein							
Additives	time (min.)	T	Exp.1 Act.	%	2 Act.	%	3 Act.	%	4 Act.	%
none	0	0°	0.43	16	0.35	12	0.32	10	0.18	6
none	45	25°	0.55	20	0.14	5	0.32	10	0.28	9
heme 1.0µM	45	25°			0.48	16	0.64	22		
10.0µM	45	25°	1.60	57			1.37	46	1.33	43
cyt.c 15.0µM	45	25°	1.88	69			0.54	18	0.98	32
heme 10.0µM	15	25°			2.94	100				
and	45	25°	2.73	100	2.94	100	2.96	100	3.11	100
cyt.c 15.0µM	45	0°			1.99	68				

Anaerobic stationary phase cells (D261),intoxicated with cycloheximide (25µg/ml) in anaerobiosis (see text) were harvested,washed and disrupted mechanically at 0°.Recombination between the native apoCcP and heme was assayed in total extract (see text). Cyt.c peroxidase activity was assayed in the S fraction.

The main observations summarized in Table II show that no holoenzyme is formed before exogenous heme (as hemin chloride dissolved in 0.1M NaOH) or cytochrome c (horse heart cytochrome c:Sigma Type VI) are added. The simultaneous presence of the two additives generally ensures a two-fold increase in the yield of CcP,ranging between 60 to 90% of the *in vivo* production by anaerobic cycloheximide-treated yeast. The most striking effect is that exogenous cytochrome c greatly increases the recombining efficiency between the native apoCcP and exogenous heme (10µM),present in

a large excess. Another basic low molecular weight protein - ribonuclease - is almost totally inefficient at a twice higher concentration.

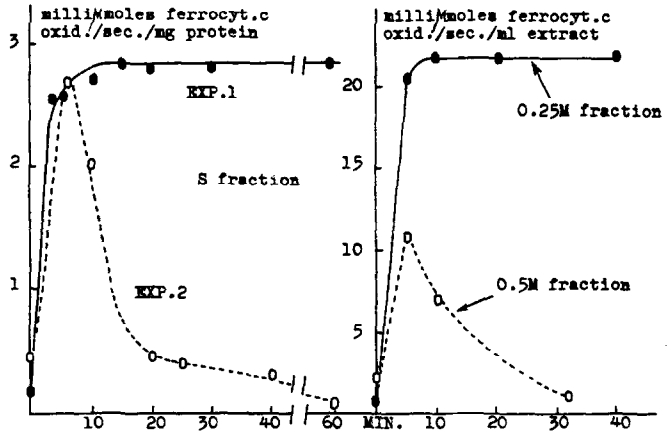

Fig. 2A and 2B - Time course of the recombination of the native apocytochrome c peroxidase with heme in enriched fractions.

S fraction obtained from anaerobic stationary phase cells (see text) (Fig.2A) was incubated at 23° with exogenous heme (10μM) and cytochrome c (15μM). After various times of incubation, cyt.c peroxidase activity (the recombined holoenzyme) was assayed spectrophotometrically (see text). (Fig.2B) - S fraction diluted with an equal volume of distilled water was adsorbed on a DEAE cellulose (Whatman DE23) column (1cmx10cm). The resin was washed with 20ml Sörensen phosphate buffer 0.05M-pH 6.0; almost all the apoCcP was eluted in a 10ml fraction with 0.25M buffer (0.25M fraction). Further elution with 0.5M buffer, removed remaining apoCcP contaminated with an inactivating factor (see text). Recombination tests of apoenzyme with heme were realized as for the S fraction.

ApoCcP, which is solubilized during the extraction procedure, is found almost quantitatively associated with the S fraction. However, the drastic conditions used make it impossible to draw any conclusion as regards the state (membrane-bound or soluble) of the native apoCcP in intact cells.

Figure 2A shows that in the unpurified fraction S, the recombination of apoCcP with exogenous heme is complete in less than 2 minutes at 23°. Although the holoenzyme, once formed, is stable at 23° in most experiments (see Exp.1), we observed in an individual case an exceptionally high rate of inactivation (Exp.2). By the use of more purified preparations (Fig.3B) derived from the S fraction after stepwise fractionation of the anionic proteins on DEAE cellulose, apoCcP becomes separated from degrading agent(s), probably proteolytic in nature.

ApoCcP partially purified on DEAE cellulose resin recombined stoe-

chiometrically with exogenous heme;on a molar equivalency basis,the concentration of the apoprotein in the anaerobic yeast cell was calculated to be close to 40 millimoles/g yeast protein (D261 strain).

Classical properties of an inducer have been proposed for oxygen (Slonimski et al,1963),the primary event during respiratory adaptation being the transformation of the presumed repressor - apoiso-2-cytochrome c - into its inactive form iso-2-cytochrome c. On the other hand we observe that cytochrome c is able to enhance the recombination efficiency of heme with the native apoCcP in crude cell-free extracts. For this reason it is tempting to propose that cytochrome c,the physiological substrate of CcP,plays a key function in vivo by unlatching the induced conversion mechanism leading to the CcP holoenzyme formation. Alternative mechanisms are being investigated as working hypotheses : (i) cytochrome c competes with some binding site of apoCcP rendering the apoprotein freely accessible to heme (ii) the recombination mechanism is only unlocked when cytochrome c has modified the spatial configuration of the native apoenzyme.

Acknowledgments -

This work was supported by the Association contract ULB-Euratom n° 016-61-10-ABIB and a "Crédit aux Chercheurs" du Fonds National belge de la Recherche Scientifique. A.A.Sels is "Chercheur Qualifié" du Fonds National belge de la Recherche Scientifique. C.Cocriamont is titular of a Bourse de Spécialisation de l'Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture.

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