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INVOLVEMENT OF IRON IN THE BIOGENESIS OF THE CYANIDE-INSENSITIVE RESPIRATION IN THE YEAST *SACCHAROMYCOPSIS LIPOLYTICA*

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SUMMARY

The involvement of iron in the biogenesis of the cyanide-insensitive respiration in the yeast *Saccharomycopsis lipolytica* has been established on the following basis: (1) endogenous metal chelation by either benzyl- or salicylhydroxamic acid, EDTA or nitrilotriacetate prevented the biogenesis of the cyanide-insensitive respiratory pathway in *S. lipolytica*. (2) Addition of Fe(III) during the biogenesis increased both the rate of the appearance of the alternative respiratory pathway and its extent. Neither Fe(II), nor Co(II), Cu(II), Al(III), La(III), Mn(II) or Mg(II) could substitute for Fe(III). (3) The biogenesis of the alternative respiratory pathway could be dissociated into two steps : (a) a first one, slow, cycloheximide-sensitive, temperature-dependent, iron-independent, leading to cells still fully cyanide-sensitive, presumably involving the de novo biosynthesis of an inactive protein moiety and (b) a second step, fast, iron-dependent, temperature-independent, cycloheximide-insensitive, leading to cells with a cyanide-insensitive respiration, presumably the activation by iron of the inactive precursor.

INTRODUCTION

Quite a large number of organisms, especially among plants and in the microbial world, display, under certain physiological circumstances, a mitochondrial alternative respiration, insensitive to cyanide or to antimycin A. The appearance of the cyanide-insensitive respiration, under natural conditions, is usually related to a phenomenon of aging or to a change in physiological state [1]. In the yeast *Saccharomycopsis lipolytica*, the cyanide-resistant respiration could be induced simply by aeration in the resting state [2].

Evidence in favor of the involvement of Fe(III) in the biogenesis of the alternative respiratory pathway is presented here.

Abbreviation: EGTA, ethyleneglycol-*bis*-(β-aminoethylether) *N,N'*-tetraacetic acid, disodium salt.

MATERIALS AND METHODS

The yeast

The yeast used in this study, *Saccharomycopsis lipolytica* [3, 4], was obtained as the strain 599 from the Centraalbureau voor Schimmelcultures (C.B.S., Baarn, the Netherlands).

Cultivation procedures

The yeast was grown in a synthetic medium (Yeast Nitrogen Base, Difco), supplemented with 1 % (w/v) glucose as sole source of carbon and energy [5]. The cells were harvested in the logarithmic phase of growth, after adjusting the pH of the broth to 6.0–6.5 by addition of 10 M KOH. Cell counts were made and dry weights determined as previously described [5].

Induction of the cyanide-insensitive respiration

Cyanide-insensitive cells of *S. lipolytica* were obtained by shaking as a 1 cm-layer, in the resting state, $1 \cdot 10^9$ cells/ml suspended either in double-distilled water or in a 1 % glucose (w/v) solution [2]. During the biogenesis of the cyanide-insensitive respiratory pathway which lasted about 1–2 h, the pH was maintained at 5.5–6.0 by addition of 1 M or 10 M KOH.

Respiration measurements

The oxygen uptakes were measured polarographically at 25 °C with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio) according to Estabrook [6]. Unless otherwise stated, the plexiglass reaction cuvette contained 4 ml of a 1 % (w/v) glucose aqueous solution and the reaction was started by addition of 100 µl of a suspension of $1 \cdot 10^9$ yeast cells/ml.

Respiration rates of whole cells are expressed as Q_{O_2}, i.e. µl O_2/h · mg dry weight [7]. The relative respiration rate : $(Q_{O_2}\,(CN^-)/Q_{O_2}) \times 100$, is the hundredfold ratio of the Q_{O_2} measured in the presence of cyanide to the Q_{O_2} measured in its absence.

Chemicals

Sodium cyanide was added to the respiration medium as an unbuffered aqueous solution [2]. Benzhydroxamic acid and salicylhydroxamic acid (Koch-Light Laboratories Ltd., Colnbrook, Bucks., England) were dissolved in dimethylformamide (Merck, Darmstadt, G.F.R.) as daily-fresh 0.4 M solutions. Up to 0.25 % (v/v) or up to 0.8 % (v/v) dimethylformamide did not affect either the respiration rates or the biogenesis of the cyanide-insensitive respiratory pathway, respectively. EDTA (Calbiochem, La Jolla, Calif), EGTA (Sigma Chemical Company, St. Louis, Mo.) and nitrilotriacetate (Titriplex I, Merck, Darmstadt, G.F.R.) were prepared as 0.4 M aqueous solutions. Cycloheximide (Actidione, Upjohn Co., Kalamazoo, (Mich.) was dissolved in ethanol at concentrations which showed no solvent effects. L-[4,5-^{3}H]-Leucine with a specific radioactivity of 64 Ci/mmol and 5′[^{3}H]uridine, with a specific radioactivity of 25 Ci/mmol, were obtained from New England Nuclear. Both were used at a final radioactivity of 2.5 µCi/$50 \cdot 10^6$ cells · ml.

TABLE I

INFLUENCE OF METAL IONS ON THE APPEARANCE OF THE CYANIDE-INSENSITIVE RESPIRATION IN WHOLE CELLS OF THE YEAST *S. LIPOLYTICA* IN THE RESTING STATE

The influence of metal ions on the appearance of the cyanide-insensitive respiration was tested on a yeast cell suspension showing in a control experiment a low level of induction. 2 mM metal ions were added to the induction medium prior to the biogenesis. The induction was carried out as described in Materials and Methods. Al(III), La(III), Cu(II) were added as chlorides. Fe(III), Fe(II), Mn(II) and Mg(II) were added as sulfates. The influence of sulfate on the appearance of the cyanide-insensitive respiration was measured, using potassium sulfate. The alternative respiration was measured in the presence of 1 mM NaCN. The biogenesis was carried out in the presence of 1 % glucose. CHX (cycloheximide) was added prior to biogenesis at a concentration of 10 mg/ml.

Shaking time (min)	Q_{O_2}											
	Control		Fe(III)		Fe(III) 0 °C		Fe(III) 20 °C+CHX		Al(III)		La(III)	
	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$
0	48.1	7.9	49.9	9.8	48.6	3.7	50.5	5.7	32.3	12.6	34.3	4.2
30	47.2	9.5	36.6	18.4	–	–	–	–	42.9	10.8	43.3	2.6
60	42.9	11	43.5	41.2	48.2	4.6	52.4	6.1	38.1	9.4	39.1	11.3
90	45.3	8.4	40.2	42.3	49	4.8	50.3	6.9	38.5	13.1	31.2	14.5

Shaking time (min)	Fe(II)		Cu(II)		Mg(II)		Mn(II)		K_2SO_4	
	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$
0	41.9	8.5	30.2	10.4	48.3	10.8	48.5	12.8	51.1	18.2
30	40.6	9.2	19.2	9.6	47.4	10.7	43.4	11.1	48.4	9.5
60	43.5	9.4	14.8	8.5	46.2	12.9	47.3	11.6	41.4	10.5
90	39.8	10.3	13.6	8.2	30.6	8.1	39	10.6	42.9	13.7

RESULTS AND DISCUSSION

When cell suspensions of $1 \cdot 10^9$ cells/ml were shaken in the resting state under inducible conditions, the amount of inducible cyanide insensitivity remained erratic. The obtained relative respiration rates varied between 0 and 90 %.

The addition of 2 mM Fe(III) increased both the rate of appearance of the cyanide-insensitive respiration and its extent. The influence of other metal ions on the biogenesis of the cyanide-insensitive respiration was also assayed. Neither Al(III), La(III), Mn(II), Mg(II) nor Fe(II), at the concentration of 2 mM, could substitute for Fe(III) as a promotor of the biogenesis. At this concentration, Cu(II) was found to inhibit the main cyanide-sensitive respiration (Table I). At non-inhibitory concentrations (20 μM) no promotion effect of the Cu(II) ion on the biogenesis could be observed.

Added Fe(III) did not promote the biogenesis of the alternative respiratory pathway at 0 °C or when cycloheximide was added to the induction medium, at concentrations which were inhibitory for the cytoplasmic protein biosynthesis [2]. The inability of added Fe(II) to promote the biogenesis of the alternative respiration in *S. lipolytica* is quite surprising, but this may be because it probably cannot be transported through the cell periplasmic membrane. The possibility that during biogenesis, which requires O_2 [2], added Fe(II) was oxidized to the ferric form, can be ruled out. As a matter of fact, the experimental conditions used for the biogenesis of the cyanide-insensitive respiration i.e. a dense suspension of $1 \cdot 10^9$ yeast cells/ml favoured the reduction of Fe(III) to Fe(II) rather than the reverse (not shown).

Metal chelation by benzhydroxamic acid decreased the level of induced alternative respiration. The dependence of the inhibition of the biogenesis of the alternative respiratory pathway on the concentration and on the nature of the metal chelator was tested with a yeast cell suspension which showed in a control experiment a high level of induction without supplementation with Fe(III). The extent of the inhibition of the biogenesis of the alternative respiratory pathway was dependent on and increased with the concentration of benzhydroxamic acid in the induction medium. Inhibitions of 30, 40, 60, 70 and 80 % were observed for concentrations in benzhydroxamic acid in induction medium of respectively 0.5, 1.0, 1.5, 2.0 and 3.0 mM after a 90-min induction period. The effect of other iron-chelators on the biogenesis of the cyanide-insensitive respiration yielded the following results: addition of sodium citrate, KCNS or EGTA to the induction medium did not prevent the appearance of the alternative respiration, whereas addition of EDTA, nitrilotriacetate or salicylhydroxamic acid to the induction medium inhibited the biogenesis (Table II).

In all the experiments performed so far, the addition of 2 mM Fe(III) always promoted the biogenesis of the alternative cyanide-insensitive respiratory pathway, whereas the addition of 3 mM benzhydroxamic acid always inhibited the biogenesis. As a matter of fact, the relative respiration rates of *S. lipolytica* averaged 16.6 with a standard deviation of 8.7 (16 experiments) before induction and 62.9 ± 20.9 (16 experiments) after induction under control conditions, i.e. without addition of either Fe(III) or benzhydroxamic acid. Whereas the relative respiration rates after induction in the presence of 3 mM benzhydroxamic acid averaged 30.6 with a standard deviation of 10.1 (9 experiments), the relative respiration rates after induction in the presence of 2 mM added Fe(III) averaged 92.2 ± 13.7 (14 experiments).

TABLE II

INFLUENCE OF IRON-CHELATORS ON THE APPEARANCE OF THE CYANIDE-INSENSITIVE RESPIRATION IN WHOLE CELLS OF *S. LIPOLYTICA* IN THE RESTING STATE

The influence of iron-chelators on the appearance of the cyanide-insensitive respiration was assayed with a yeast cell suspension showing in a control experiment a high level of biogenesis in the absence of added Fe(III). Inductions were carried out as described in Materials and Methods in the presence of various iron-chelators added to the induction medium prior to the biogenesis. Final concentrations of BHAM (benzhydroxamic acid) : 2 mM; SHAM (salicylhydroxamic acid): 3 mM; EDTA: 1.5 mM; NTA (nitrilotriacetate): 2 mM; EGTA: 2 mM; sodium citrate: 5 mM; KCNS: 2 mM and Fe(III): 2 mM $Fe_2(SO_4)_3$. The alternative respiration was measured in the presence of 1 mM NaCN.

Shaking time (min)	Q_{O_2}									
	Control		BHAM		SHAM		EDTA		NTA	
	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$
0	37.0	7.9	46.9	5.7	36.2	8.8	41.8	9.6	37.4	6.9
90	32.9	26.7	33.2	7.6	37.2	11.5	34.1	14.5	41.6	17.6

Shaking time (min)	EGTA		Citrate		KCNS		Fe(III)	
	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$
0	37.3	9.9	47.7	0	51	0	42.6	9.0
90	36.4	31.1	36.4	26.1	41	46	38.7	40.2

The possibility that the addition of chelators to the induction medium resulted in the inhibition of the measured oxygen uptakes by the alternative respiratory pathway, having been synthesized, could be ruled out. As a matter of fact, up to 3 mM metal chelator were added to a suspension containing 10^9 yeast cells/ml. Both metal chelator and yeast cells were diluted 40-fold in the respiration chamber (see Materials and Methods). It could be shown that concentrations of metal chelators up to 75 μM had no effect on the rate of oxygen uptake by $25 \cdot 10^6$ yeast cells/ml. Furthermore, in a control experiment, when a suspension of fully cyanide-insensitive yeast cells, at a concentration of $1 \cdot 10^9$ cells/ml, was shaken, under inducible conditions, either in the presence of 3 mM benzhydroxamic acid or 2 mM added Fe(III), no effect of benzhydroxamic acid or Fe(III) on the measured rates of oxygen uptake could be observed.

That benzhydroxamic acid did not hamper the overall protein biosynthesis in the biogenesis was demonstrated in the following way. The incorporation of L-[^{3}H]-leucine and [^{3}H]uridine in whole cells of *S. lipolytica*, in the resting state, was followed as a function of time in a $50 \cdot 10^6$ cells/ml suspension, according to the filter-paper discs method described by Mans and Novelli [8]. 2 mM benzhydroxamic acid did not affect the incorporation of [^{3}H]-uridine in a significant manner and did not prevent

TABLE III

REVERSION BY IRON (III) AT ROOM TEMPERATURE, AT 0 °C OR IN THE PRESENCE OF CYCLOHEXIMIDE, OF THE INHIBITORY EFFECT OF BENZHYDROXAMIC ACID ON THE BIOGENESIS OF THE ALTERNATIVE RESPIRATION IN *S. LIPOLYTICA*

$1 \cdot 10^9$ yeast cells/ml were shaken, under inducible conditions, in the presence of either 2 mM benzhydroxamic acid or 2 mM added Fe(III), at room temperature. After 90 min, the cells incubated in the presence of benzhydroxamic acid were divided in three batches. 2 mM Fe(III) was added (a) to the first one, maintained at room temperature, (b) to the second one, precooled at 0 °C and (c) to the third one, maintained at room temperature, but to which 10 mg cycloheximide/ml had been added 15 min previously. Thereupon, the incubation under inducible conditions was resumed for 25 min. The alternative respiration was measured in the presence of 1 mM NaCN.

Shaking time (min)	Q_{O_2}					
	BHAM				Fe(III)	
	−NaCN	+NaCN			−NaCN	+NaCN
0	36.8	6.2			37.4	5.7
45	37.0	6.6			33.5	17.3
90	37.8	8.5*			34.4	27.6
Time after addition of iron (min)	Addition of 2 mM Fe(III) to the induction medium containing BHAM					
	20 °C		0 °C		20 °C+CHX	
	−NaCN	+NaCN	−NaCN	+NaCN	−NaCN	+NaCN
0	36.9	8.8	34.4	7.2	38.7	9.6
5	35.6	16.4	35.7	9.1	35.5	14.5
15	35.9	16.8	35.0	12.8	36.5	16
25	37.2	17.0	36.9	15.7	37.1	17.2

* This value did not change significantly during the next hour.

the overall incorporation of L-[^{3}H]-leucine, although it reduced its level by about 10 %, in a reproducible way.

The nature of the involvement of iron in the biogenesis of the cyanide-insensitive respiration could be further narrowed down by dissociating this biogenesis into two steps. In a first step, the yeast cells were preincubated, under inducible conditions, in the presence of benzhydroxamic acid, at concentrations which prevented the appearance of the cyanide-insensitive respiration, for a length of time equal to that usually required for a complete biogenesis. In a second step, further addition of Fe(III) to these preincubated cells did promply promote the appearance of the alternative cyanide-insensitive respiration, even at 0 °C or in the presence of cycloheximide, at doses inhibitory for cytoplasmic protein biosynthesis (Table III). This experimental data are best interpreted in the following way. The first step, slow (requiring 1 h), sensitive to cycloheximide, an inhibitor of cytoplasmic protein biosynthesis [9], at doses barely sufficient to prevent growth of *S. lipolytica* [2] or incorporation of L-[^{3}H]-leucine (not shown), temperature-dependent (absent at 0 °C) and iron-independent, leading to cells still cyanide-sensitive, presumably consisted i.a., of the de novo biosynthesis of an inactive protein moiety, precursor of an alternative respiratory pathway component. The second step, fast (requiring 5 min), relatively temperature-independent (approximately 15 min at 0 °C), cycloheximide-insensitive, iron-dependent, leading to cells insensitive to cyanide, presumably consisted in the fixation of iron on the inactive protein moiety to yield an active alternative respiratory pathway component.

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