COMPARISON OF OXIDATION RATES OF REDUCED PYRIDINE NUCLEOTIDES BY NORMAL AND RESPIRATION DEFICIENT MUTANT YEAST

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Slonimski and Hirsch (1) have measured the activities of some enzymes of the glycolytic, tricarboxylic acid cycle and electron transport mechanisms in normally-respiring baker's yeast (Saccharomyces cerevisiae) and in a respiration-deficient mutant yeast. Their findings indicated the mutant respiration-deficient yeast lacked cytochrome oxidase, succinic dehydrogenase cytochrome b complex and DPNH cytochrome c reductase. Only the cytochrome c component was present in normal concentrations in both yeasts. The present study was confined to examination of the DPNH and TPNH oxidation systems in both normally-respiring baker's yeast and the above mentioned mutant yeast in the presence and absence of antimycin A.

METHODS

Preparation of cell-free extracts. The cells of the normal yeast (obtained from C.C. Lindegren) and mutant yeast (obtained from B. Ephrussi) were grown in a yeast extract (0.5%), dextrose (1%) and peptone (0.3%) medium in a shaker for two days and were then centrifuged and washed three times with glass distilled water. Following centrifugation the cells were frozen in a deep freeze and stored for several days. The frozen cells were thawed as needed for the individual experiments and placed in the fly press described by Hughes (2) and refrozen with liquid nitrogen. The cells were crushed in this press and the crushed preparations were centrifuged first in the Servall SS-1 centrifuge for 10 to 15 minutes to remove cellular debris and the supernatants to which 4 to 5

This work was performed in the laboratory of the late E.S.G. Barron, Department of Medicine, University of Chicago.

volumes of 0.05 M phosphate buffer of pH 7.35 were added, were then centrifuged for 30 minutes in the Spinco preparative centrifuge at 40, 000 rpm (105, 400 x G) using rotor No. 40. The precipitates were washed with an equal amount of this phosphate buffer and the suspensions were recentrifuged in the Spinco as above. The resultant precipitates were then mixed with 1/5 of their volumes of phosphate buffer and these were throughly mixed until the suspensions were homogeneous and could be pipetted with ease.

Assay procedures. Examinations of the absorption spectra of the two cell-free extracts were performed after reduction by sodium thiosulfate (dithionite). It was noted that both normal and mutant yeast cell-free extracts had absorption peaks at 549 - 552 mμ. Since ferrocytochrome c is well known to have its alpha band absorption peak in this region, increase in optical density (O.D.) measured at 549 mμ was used to indicate formation of ferrocytochrome c and the value given by Massey (3) of 21 $cm^2/\mu M$ at 550 mμ was used for calculations of mμM quantities.

The activities of mutant cell-free extracts toward TPNH oxidation were also assayed by noting the decrease in O.D. at 340 mμ. At this wavelength, DPNH, TPNH and cytochrome c all have appreciable absorptions which are different for the reduced and oxidized forms. The specific extinction coefficient for TPNH minus TPN at 340 mμ is 6.22 $cm^2/\mu M$ (4) while that estimated from Margolaish (5) for ferricytochrome c minus ferrocytochrome c is approximately 5.0 $cm^2/\mu M$. Thus, at 340 mμ the oxidation of 1 μM of TPNH (or DPNH) plus the concomitant formation of 2 μM of ferrocytochrome c would result in a total decrease of O.D. of approximately 16.22 cm^2. Therefore, $\frac{6.22}{16.22}$ X $\frac{\Delta O.D.\ (340\,m\mu)}{6.22} = \frac{\Delta O.D.\ (340\,m\mu)}{16.22}$ = μM of TPNH was used to calculate the amount of TPNH oxidized and $\frac{10}{16.22}$ X $\frac{\Delta O.D.\ (340\,m\mu)}{5} = \frac{\Delta O.D.\ (340\,m\mu)}{8.11}$ = μM of ferricytochrome c was used to calculate the amount of ferricytochrome c which was reduced.

RESULTS

Normal yeast and mutant yeast cell-free extracts were tested for their ability to reduce cytochrome c with DPNH or TPNH as substrate in the presence

and absence of antimycin A. Table I. shows the results of these experiments. It must be emphasized that no attempt was made to inhibit cytochrome oxidase since the comparable system in mutant yeast which lacked cytochrome oxidase was partially inhibited by cyanide as shown in Table II. Thus, the rates of cytochrome c reduction obtained here most probably represent only partial activities of these systems.

TABLE I.

Activities are expressed as mμM of ferrocytochrome c formed/min./0.05 ml of cell-free extract as measured at 549 mμ.

	DPNH as substrate		TPNH as substrate	
	Normal Yeast	Mutant Yeast	Normal Yeast	Mutant Yeast
Without antimycin A	74.8	1.9	30.0	7.4
With 1.2 μgm/ml antimycin A	10.5	1.6	18.6	7.0

All reactions were performed in a total volume of 1 ml in cuvettes having a 1 cm light path and the assays were recorded using the Beckman DK-2 recording spectrophotometer. The reaction vessels contained the following: Cytochrome c, 1 mg; DPNH or TPNH, 0.125 μM; antimycin A, 1.2 μgm; cell-free extract, 0.05 ml; phosphate buffer 0.045 M, pH 7.35 to 1 ml. Normal yeast cell-free extract was diluted 1:10 prior to assay and the mutant yeast cell-free extract was assayed directly.

In normal yeast it can be seen that the bulk of DPNH oxidation with concomitant cytochrome c reduction occurs via an antimycin A sensitive pathway. Only 14% of the total activity appears antimycin A insensitive (at a concentration of 1.2 μgm/ml). The total TPNH oxidation rate by normal cell-free extracts was approximately 40% as rapid as the total DPNH oxidation rate. However, the antimycin A insensitive system was 177% as active as its DPNH counterpart. In contrast, it can be seen in Table I. that mutant yeast cell-free extracts demonstrated reduced oxidation rates of both DPNH and TPNH (as measured, again, by cytochrome c reduction) and that both systems were only slightly affected by antimycin A.

The formation of ferrocytochrome c by cell-free extracts of mutant yeast with TPNH as substrate was also measured in the presence of KCN (0.9 μM/ml) as noted in Table II. The KCN produced an inhibition of 56% in the rate of ferro-

cytochrome c formation and this was decreased to only 29% inhibition by addition of 0.01 mg/ml flavin mononucleotide (FMN). Increasing the FMN to 0.03 mg/ml failed to increase the rate above that obtained with 0.01 mg/ml.

TABLE II.

Assay of mutant yeast cell-free extract with TPNH as substrate. Activities are expressed as mμM/min./0.05 ml of mutant yeast cell-free extract. Conditions and reactants as in Table I. (without antimycin A) plus the addition of 0.9 μM of KCN and 0.01 mg FMN.

	Assayed at 549 mμ	Assayed at 340 mμ	
	Ferrocytochrome c formed	Ferrocytochrome c formed	TPNH consumed
With KCN	3.24	5.18	2.59
With KCN plus FMN	5.24	5.92	2.96

The above two experiments were also assayed by following the decrease in O.D. at 340 mμ. From Table II., it can be seen that in the presence of KCN and FMN, results obtained utilizing both the 340 mμ and 549 mμ wavelengths compare favorably, whereas the results obtained in the presence of KCN but in the absence of FMN do not compare favorably.

DISCUSSION

The results of these experiments demonstrate the presence in normal yeast cell-free extracts of an antimycin A sensitive DPNH oxidase system which is linked at least in part to cytochrome c. Although the major part of the DPNH oxidation with concomitant cytochrome c reduction occurs via the antimycin A sensitive pathway, an appreciable amount of antimycin A insensitive activity still remains at concentrations of 1.2 μgm of antimycin A/ml. Ramachandran and Gottlieb (6), obtained almost complete inhibition of DPNH oxidation in cell-free extracts of another strain of baker's yeast (S. cerevisiae) using a concentration of only 0.1 μgm/ml of antimycin A. These workers also concluded in another report (7) that the oxidation of pyridine nucleotide probably proceeds solely via a flavoprotein system through coenzyme Q or cytochrome b, on to an antimycin A-ascosin sensitive factor and thence to a cytochrome c_1-cytochrome c system. In their scheme there is no allowance for a DPNH specific

cytochrome c reductase system which is insensitive to antimycin A. Presumably their cell-free extracts contained both mitochondria and microsomes. Martin and Morton (8) working with Beta vulgaris found a DPNH cytochrome c reductase in mitochondria which was entirely inhibited by 0.006 μM antimycin A. They considered this enzyme to be a genuine cytochrome c reductase. Their microsomal fractions, however, contained a DPNH cytochrome c reductase which was insensitive to 3 μM of antimycin A. They concluded that the physiological function of this microsomal enzyme had nothing to do with cytochrome c. The cell-free fractions used in the present experiments presumably contained both mitochondria and microsomes since the cell-free extracts were subjected to forces of 105, 400 x G for 30 minutes.

Assuming the non-existence of a specific TPNH-diaphorase (the only one described to date was in plant tissue (10)), the antimycin A sensitive TPNH oxidation presumably proceeds via the pyridine nucleotide transhydrogenase (11) to form DPNH and thence to the DPNH linked diaphorase. If this is, indeed, the case, then the pyridine nucleotide transhydrogenase would be the rate-limiting reaction since the remainder of the system is much more rapid as seen in Table I. The greater part of the TPNH oxidation (about 60%) thus proceeds via the TPNH specific cytochrome c reductase.

The very low activity of DPNH cytochrome c reductase in the mutant yeast shown in Table I. is consistent with the previous findings of Slonimski and Hirsch (1). It is also consistent with the experiments of Slonimski (12) on normal yeast grown anaerobically in which the activity of DPNH cytochrome c reductase is very little but which increases 40-fold upon exposure to oxygen. If this enzyme system is considered to exist essentially for energy purposes as suggested by Horecker (13) then it logically follows that anaerobically grown yeast would probably possess small amounts of this enzyme and aerobically grown yeast would contain large amounts. Comparison of the DPNH oxidation systems with the TPNH oxidation systems reveals the decreased vulnerability of the TPNH systems to levels of oxygen tension and this would also be in agreement with Horecker's suggestion that the TPNH system exists mainly for synthetic purposes and thus would be comparatively insensitive to levels of oxygen tension.

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