Studies on the Mitochondrial Electron Transport System in *Aspergillus*[1] (34491)

EDWIN S. HIGGINS AND WILSON H. FRIEND

Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23219

Mitochondria from wild and nitroaryl-resistant strains of *Aspergillus niger* have been isolated and have provided evidence toward explaining the different metabolic capabilities of these two strains (1). Mitochondria from the resistant strain respired more vigorously and had a higher degree of phosphate-acceptor control. Although NADH was oxidized most rapidly of all externally supplied substrates by the organelles, the major respiratory chains were similar to those in mitochondria from higher organisms. The present communication describes activities of individual segments of the respiratory chains and indicates that oxidation of external NADH is probably not coupled to phosphorylation.

Materials and Methods. Isolation of the *A. niger* strains, culture methods, isolation of mitochondria, protein assay, and source of chemicals were all as described previously (1).

Assays of cytochrome *c* reduction were conducted with a Gilford recording spectrophotometer thermally controlled at 30°. Absorbance changes at 550 mμ were traced in a mixture of 0.5 *M* mannitol, 0.5 m*M* EDTA, 5.26 m*M* KCl, 5.26 m*M* potassium phosphate, 0.13% dialyzed crystalline bovine serum albumin (BSA), 40 μ*M* ferricytochrome *c*, 6 m*M* KCN, and mitochondria (2–2.5 mg of protein), pH 7.8. Reaction was started by addition of 167 μ*M* succinate or NADH.

The various oxidase activities were assayed at 30° with a model 53 biological oxygen monitor and a Clark fixed-voltage polarographic probe (Yellow Springs Instrument Co.). A 100 mV potentiometric recorder provided a continuous tracing of O_2 concentration with time. Cytochrome *c* oxidase was measured in a mixture of 0.25 *M* sucrose, 6.67 m*M* potassium phosphate, 13.3 μ*M* cytochrome *c*, 0.13% BSA, 2–2.5 mg mitochondrial protein, and either 1 m*M* *N*, *N*, *N'*,*N'*-tetramethylparaphenylenediamine (TMPD) or 2.5 m*M* ascorbate, pH 7.8. Activities were corrected for minor autooxidation of substrates. The complete respiratory chains for NADH and for succinate were assayed in a mixture of 0.25 *M* sucrose, 6.67 m*M* potassium phosphate, 0.13% BSA, 2-2.5 mg mitochondrial protein, and NADH or succinate as indicated, pH 7.8. Similar results obtained in all these polarographic determinations when sucrose was replaced by 0.5 *M* mannitol, 0.5 m*M* EDTA, and 5.26 m*M* KCl. State 4:state 3:state 4 transitions were produced by addition of 0.640 μmole of ADP.

The conventions used in naming respiratory states, respiratory control, and ADP/O ratios are those of Chance and Williams (2).

Results and Discussion. The lower respiratory rate of mitochondria from the wild strain (1) was clearly reflected in the succinate-cytochrome *c* reductase activities; however differences in the NADH-cytochrome *c* respiratory segments of the two strains were not so marked (Table I). Both malonate, by inhibition of succinate dehydrogenase, and antimycin, by inhibition at the cytochrome b-c_1 site, depressed the succinate-cytochrome *c* reductase portion of the chain in mitochondria from both organisms. Although cyanide and azide were essentially equally effective in preventing loss of electrons via cytochrome oxidase, preincubation of the preparations

[1] Supported in part by National Science Foundation Grant GB-5886, Division of Biological and Medical Sciences.

TABLE I. Comparison of Cytochrome *c* Reductase Activities in Mitochondria from Two Strains of *A. niger*.

Respiratory segment	Additions	Wild type	Resistant strain
Succinate-cytochrome *c* reductase	None	26.9 ± 2.1[a]	44.2 ± 3.0
	167 μM malonate	11.6 ± 0.9	7.2 ± 0.5
	333 μM malonate	1.1 ± 0.1	0.7 ± 0.5
	Antimycin[b]	6.2 ± 0.3	14.3 ± 3.7
	6 mM NaN_3[c]	36.5 ± 1.3	51.4 ± 3.5
	Idem preincubated[d]	134.7 ± 3.0	223.0 ± 2.8
NADH-cytochrome *c* reductase	None	234.9 ± 8.1	280.5 ± 9.0
	7 μM rotenone	89.4 ± 6.7	114.4 ± 5.4
	10 mM amytal	58.5 ± 5.6	66.1 ± 4.3
	10 mM seconal	30.6 ± 2.1	34.4 ± 2.9
	Antimycin[b]	58.7 ± 4.9	63.5 ± 4.5
	6 mM NaN_3[c]	575.0 ±20.3	518.4 ±17.4
	Idem preincubated[d]	959.7 ±19.7	929.4 ±17.8

[a] ΔA_{550} min^{-1} g mitochondrial protein^{-1} ± SEM for three mitochondrial preparations.
[b] 0.8 mμmole/mg mitochondrial protein.
[c] Cyanide was omitted from the medium.
[d] Preincubation was for 10 min at 0° plus 5 min at 30° before addition of substrate.

with azide resulted in considerable stimulation of cytochrome *c* reduction by succinate. This behavior was not observed with cyanide. The level of cyanide used completely blocked cytochrome oxidase.

Inhibitors of the NADH dehydrogenase portion (rotenone, amytal, seconal) and of the cytochrome *b*-c_1 portion (antimycin) both depressed NADH-cytochrome *c* reductase activity of mitochondria from both strains (Table I). This latter activity, unlike succinate-cytochrome *c* reductase, was considerably higher when terminal disposal of electrons was blocked by azide than by cyanide. In common with succinate-cytochrome *c* reductase, the rate of cytochrome *c* reduction by NADH was further augmented by preincubation with azide, but not with cyanide. Azide, beside inhibiting cytochrome oxidase, acts as an uncoupling agent; the latter activity, according to Wojtczak (3) occurs predominantly at the first phosphorylation site. Thus, the effects of cyanide and of azide (without preincubation) were comparable in the succinate-cytochrome *c* reductase assay since phosphorylation site I is not a component of this complex. However, NADH-cytochrome *c* reductase activity was much higher with azide than with cyanide (even though cyanide completely blocked cytochrome oxidase) and we view this increase as an expected stimulation by uncoupler of state 4 respiration across site I. In addition, the increase in both reductase activities by preincubation with azide implies a further action of azide, possibly at site II.

Cytochrome oxidase activity (Table II) of resistant strain mitochondria was considerably higher than that of the wild type, and the respiratory control ratio (RCR) for this activity did not exceed 1.22 in any mitochondrial preparations (not shown in Table). Since resistant strain mitochondria oxidizing

TABLE II. Comparison of Cytochrome *c* Oxidase Activities.

Electron donor	Additions	Wild type	Resistant strain
1 mM TMPD	None	263 ± 23[a]	424 ± 39
	6 mM KCN	0	0
	6 mM NaN_3	18 ± 2	33 ± 5
	Antimycin[b]	278 ± 24	558 ± 41
2.5 mM Ascorbate	None	70 ± 4	177 ± 8
	6 mM NaN_3	19 ± 1	31 ± 1
	Antimycin[b]	84 ± 4	185 ± 10

[a] Millimicroatoms O_2 min^{-1} mg mitochondrial protein^{-1} ± SEM for three mitochondrial preparations.
[b] 0.8 mμmole/mg mitochondrial protein.

TABLE III. Comparison of NADH Oxidase and Succinoxidase Velocities and Respiratory Control in Mitochondria from Two Strains of *A. niger*.

Substrate	Wild type			Resistant strain		
	State 3	State 4	RCR	State 3	State 4	RCR
0.35 m*M* NADH	19.1 ± 0.4[a]	19.1 ± 0.4[a]	1.00[b]	67.8 ± 1.1	66.7 ± 1.5	1.02
0.35 m*M* Succinate	12.4 ± 0.3	10.0 ± 0.4	1.24	53.6 ± 5.1	36.0 ± 5.4	1.54
3.50 m*M* Succinate	23.7 ± 2.2	17.3 ± 1.2	1.37	109.4 ±14.2	37.0 ± 4.9	2.96

[a] Millimicroatoms O_2 min^{-1} mg mitochondrial $protein^{-1}$ ± SEM for three mitochondrial preparations.

[b] Respiratory control ratio = state 3 rate/state 4 rate.

succinate, for example, are characterized by RCR's in excess of 3.0 (1), it appears that the third phosphorylation site in mitochondria from *A. niger*, as from other sources (4), has relatively minor significance on the overall control of respiration by phosphate-acceptor. Oxygen was reduced nonenzymically by ascorbate plus cyanide at a vigorous rate. (The oxygen content of the medium was completely depleted in about 1 min.) Autooxidation of ascorbate alone was only 37 mμ atoms min^{-1}, and cyanide alone did not reduce oxygen. Therefore, inhibition by cyanide of ascorbate-cytochrome oxidase activity was not studied. This problem was not encountered with ascorbate plus azide. Although ascorbate is a much more sluggish reductant of ferricytochrome *c* than is TMPD, the two were not used in tandem in these studies in order that cyanide inhibition could be examined. Thus, substrate quantities of TMPD were used so that ascorbate addition was unnecessary. The minor stimulation of cytochrome oxidase activity by antimycin we ascribe to prevention of the energy-dependent reversed electron transport from the terminal segment of the respiratory chain (5).

The higher respiratory activity of the complete chains for NADH and for succinate oxidation in mitochondria from the resistant strain is apparent in Table III. Oxidation of 0.35 mM NADH commenced immediately upon its addition, but on addition of an equimolar quantity of succinate there was a 30 sec delay in the state 1:state 4 transition. On increasing the succinate concentration 10-fold this delay disappeared. We take this to mean that NADH is taken up more rapidly than succinate by these mitochondria. However, oxidation of externally supplied NADH was not subject to control by phosphate-acceptor. Indeed, RCR = 1.00 for NADH-cytochrome *c* reductase activity also in these mitochondria (not shown in Table I). We have no explanation at this time for the observed dependence of the degree of respiratory control on the succinate concentration although the same phenomenon has been observed in mouse liver mitochondria, and it was suggested that hydrogen pressure produced by dehydrogenase activity (*i.e.*, substrate concentration) is the factor which determines the degree of respiratory stimulation by ADP (6). Evidence has been presented for locating a rate-limiting site for NADH oxidation on the substrate side of cytochrome *c* in these organelles (1). Rates of cytochrome *c* oxidation in mitochondria from both strains were much higher than were their respective maximal velocities for succinate and NADH oxidation (compare Tables II and III). Therefore, at least under the specified conditions, it seems reasonable to place the rate-limiting site for succinate oxidation also on the substrate side of cytochrome *c*.

Structurally and functionally intact mitochondria from several sources oxidize external NADH (7–9). Those described here differ from the *A. niger* mitochondria studied by Watson and Smith (10), but are similar to avian heart mitochondria (7) in that mitochondrial oxidation of external NADH appeared not to be coupled to phosphorylation nor to be subject to phosphate-acceptor con-

trol. The coupled oxidation of NADH by *A. niger* mitochondria observed by Watson and Smith was insensitive to rotenone and amytal and they suggested that the oxidation was via a nonphosphorylating pathway between NADH and cytochrome *b*. The remaining phosphorylating sites between cytochrome *b* and oxygen could account for their observed ADP/O ratios of 1.4-1.8 with NADH. Similar results were obtained with yeast mitochondria (9). Our experiments are in concurrence with a nonphosphorylating path between exogenous NADH and cytochrome *b* (RCR = 1.00 for NADH-cytochrome *c* reductase) but we must also propose a totally nonphosphorylating route for external NADH since RCR = 1.00 and ADP/O = 0 for NADH oxidase activity of mitochondria from both strains. Evidence has already been presented that functionally and structurally intact mitochondria from *A. niger* promote the vigorous oxidation of exogenous NADH (1), but that this activity is rotenone- and amytal-sensitive. It may be possible that these differences in the coupling of NADH oxidation to phosphorylation and in the rotenone-amytal sensitivity may be a consequence of the different strains of *A. niger* used by Watson and Smith (van Tieghem) and by us (Mulder), or of the different pH of assay (6.5 *vs* 7.8).

Earlier investigations had established differences in subcellular localization of carbohydrate oxidation during growth in wild and nitroaryl-resistant organisms. That is, catabolism of carbohydrate was predominantly via the direct oxidative pathway in wild type *A. niger* and via the citrate cycle in the resistant strain (11, 12). The argument that these major metabolic diversities may be referable to dissimilarities in mitochondrial function (1) now gains further substantiation in view of the higher electron transport efficiencies of the various complexes, as well as of the complete respiratory chains, and associated respiratory control ratios in mitochondria from the resistant strain.

Summary. Mitochondria from two strains of *Aspergillus niger* were examined for succinate-cytochrome *c* reductase, NADH cytochrome *c* reductase, cytochrome *c* oxidase, succinoxidase, and NADH oxidase activities, and for phosphate-acceptor control. Except for NADH-cytochrome *c* reductase, each of these activities was considerably higher in the nitroaryl-resistant strain than in the wild type. These data supplied further evidence of augmented mitochondrial function in the resistant strain which aids in explaining metabolic differences between the two. Although intact *Aspergillus* mitochondria oxidize exogenous NADH, this activity did not appear to be coupled to phosphorylation.

1. Higgins, E. S. and Friend, W. H., Can. J. Biochem. **46**, 1515 (1968).

2. Chance, B. and Williams, G. R., Advan. Enzymol. **17**, 65 (1956).

3. Wojtczak, L., *in* "Regulation of Metabolic Processes in Mitochondria" (J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, eds.), p. 179. Elsevier, New York (1966).

4. Hatefi, Y., Jurtshuk, P., and Haavik, A. G., Arch. Biochem. Biophys. **94**, 148 (1961).

5. Klingenberg, M., *in* "Biological Oxidations" (T. P. Singer, ed.), p. 45. Wiley, New York (1968).

6. Cereijo-Santaló, R., Can. J. Biochem. **43**, 425 (1965).

7. Blanchaer, M. C., Lundquist, C.-G., and Griffith, T. J., Can. J. Biochem. **44**, 105 (1966).

8. Cereijo-Santaló, R., Can. J. Biochem. **44**, 67 (1966).

9. Onishi, T., Kawaguchi, K., and Hagihara, B., J. Biol. Chem. **241**, 1797 (1966).

10. Watson, K. and Smith, J. E., Biochem. J. **104**, 332 (1967).

11. Higgins, E. S. and Chambers, W. G. Enzymologia **30**, 265 (1966).

12. Higgins, E. S. and Chambers, W. G., Arch. Biochem. Biophys. **103**, 87 (1963).

Received Sept. 8, 1969. P.S.E.B.M., 1970, Vol. 133.