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Adenosine 5'-Monophosphate-Stimulated Cyanide-Insensitive Respiration in Mitochondria of *Moniliella tomentosa*

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Mitochondria of the yeastlike fungus *Moniliella tomentosa* oxidize reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate, succinate, isocitrate, and lactate. These oxidations are completely inhibited by cyanide or antimycin A in mitochondria isolated from cells grown in the standard medium. On the other hand, the oxidation of all substrates, except lactate, is almost completely insensitive to cyanide or antimycin A in mitochondria from cells grown in the presence of ethidium bromide. In this instance, the oxidation is mainly mediated by an alternate oxidase which can be blocked by salicyl hydroxamic acid. The alternate oxidase can be specifically stimulated by adenosine 5'-monophosphate and this provides a new method for the characterization of the alternate oxidase in mitochondria of *M. tomentosa*.

Moniliella tomentosa is an osmotolerant yeastlike fungus (6). Growth of the organism in media of high sugar concentration results in the biosynthesis of large amounts of ethanol or the polyols glycerol and erythritol (9, 11).

Several factors affect the ratio of ethanol to polyols produced by osmotolerant yeasts (for review see reference 22). Especially interesting is the shift from ethanol to polyol production under conditions of increased aeration. As it is not known why osmotolerant yeasts form polyols under these conditions, a study of the respiratory system of *M. tomentosa* was undertaken (10). It was found that the respiration of whole cells can be mediated by a branched respiratory system. One system consists of a normal respiratory chain that is mediated by *a*-, *b*-, and *c*-type cytochromes sensitive to cyanide (CN) and antimycin A. The other system is insensitive to these inhibitors but can be specifically blocked by salicyl hydroxamic acid (SHAM). The activity of the CN-insensitive respiration depends on the age of the cells and can be greatly increased by growing the cells in the presence of acriflavine, ethidium bromide (EBr), chloramphenicol, lincomycin, or antimycin A.

It has been shown that a SHAM-sensitive oxidase also occurs in other organisms and that it is localized in the mitochondria (7, 13, 16, 23). The present work was carried out to localize the SHAM-sensitive respiration in *M. tomentosa* and to study some of its characteristics. It was found that the alternate respiratory system is present in the mitochondrial fraction of the cells and that it could be specifically stimulated by adenosine 5'-monophosphate (AMP), thus providing a new parameter for the study of this system in *M. tomentosa*.

MATERIALS AND METHODS

Growth of the organism. *M. tomentosa* var. *pollinis* (6) was grown at 30 C on a reciprocal shaker in 500-ml Erlenmeyer flasks containing 50 ml of medium. The aeration rate corresponded to a sulfite oxidation rate of 22 mmol/liter per h as determined by the method of Cooper et al. (4). The standard medium contained glucose (10%), yeast extract (1%), and urea (0.1%). Appropriate amounts of a 0.5% stock solution of EBr, sterilized by filtration, were added to the media just before inoculation when the cells were grown in the presence of this drug. The final concentration of EBr in the media was 10 μg/ml.

Preparation of mitochondria. Mitochondria were prepared from cells grown for about 25 h in the standard medium (normal mitochondria) or from cells grown for about 35 h in the presence of EBr (EBr mitochondria). The cells were harvested by filtration of the media on a large Buchner funnel, washed with water, and then blotted dry between two sheets of Whatman no. 1 paper. About 100 g (wet weight) of cells were suspended in 100 ml of 0.33 M sucrose, 1 mM ethyleneglycol-bis-(β-amino-ethyl ether) *N,N'*-tetraacetic acid (EGTA), 0.3% bovine serum albumin (BSA) and 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0) at 2 C. This suspension was passed through a precooled French pressure cell at 1,000 lb/in². The resulting extract was brought to pH 7.2 with N NaOH and centrifuged at 1,000 × *g* for 10 min. The supernatant was incubated for 30 min at 2 C in the presence of 20 mg α-amylase (170 U/mg) and then centrifuged at 1,200 × *g* for 20 min. The

pellet was mixed with 60 ml of 0.33 M sucrose, 1 mM EGTA, 0.3% BSA, and 10 mM Tris-hydrochloride buffer (pH 7.2), and then homogenized in a loosely fitting Teflon-glass Potter homogenizer. The suspension was again centrifuged at 500 × *g* for 8 min to remove remaining cell debris and then at 10,000 × *g* for 15 min to obtain the mitochondrial fraction. The mitochondria were washed in the 10 mM Tris-hydrochloride buffer (pH 7.2) and finally homogenized by hard pipetting to give a suspension of particles containing about 25 to 50 mg of protein per ml.

Respiratory measurements. Mitochondrial oxygen uptake rates were determined at 25 C with a Clark oxygen electrode, using 0.5 to 1.0 mg of mitochondrial protein in 3 ml of air-saturated buffer. The respiration buffer consisted of 0.3 M sucrose, 0.7 mM ethylenediaminetetraacetic acid and 8 mM Tris-hydrochloride (pH 7.2). The concentration of the substrates used was 0.5 mM for reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH), 5 mM for pyruvate, 10 mM for malate, α-ketoglutarate, isocitrate, or lactate and 20 mM for succinate. For the experiments with α-ketoglutarate or pyruvate, malate (1 mM) was included in the reaction mixture. Calculation of the oxygen uptake was based on a dissolved oxygen concentration of 240 μmol of O_2 per liter (2). The respiratory rates are expressed as nanoatoms of oxygen consumed per minute and per milligram protein.

Determination of enzyme activities. Malate dehydrogenase, myokinase, and adenosine triphosphatase (ATPase) were measured by coupling the reactions to the oxidation or the reduction of pyridine nucleotides and monitoring spectrophotometrically the change in optical density at 340 nm. The extinction coefficient used for the calculation of the specific activities was taken to be 6.22 mM^{-1}/cm. Malate dehydrogenase was measured by following the oxidation of NADH in a mixture consisting of the respiration buffer, KCN (1 mM), *cis*-oxaloacetic acid (10 mM), NADH (0.2 mM), and mitochondria. Myokinase was determined by the reduction of oxidized nicotinamide adenine dinucleotide phosphate (NADP) in the presence of glucose, adenosine 5'-diphosphate (ADP), hexokinase, glucose 6-phosphate dehydrogenase and $MgCl_2$ according to Schnaitman and Greenawalt (15). ATPase was measured by coupling the reaction to the oxidation of NADH in a system containing respiration buffer, adenosine 5'-triphosphate (ATP), phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, and $MgCl_2$ according to Chan et al. (1).

Analytical methods. Proteins were determined by the method of Lowry et al. (14) in the presence of 0.2% sodium deoxycholate. BSA was used as the standard. The concentrations of nucleotides and antimycin A were estimated spectrophotometrically using the extinction coefficients given by Sober (21) and Slater (20).

Materials. The following materials were obtained commercially: NADH, NADPH, *cis*-oxaloacetic acid, DL-isocitrate, BSA (type V), atractyloside (potassium salt), oligomycin, and EBr from Sigma Chemical Co. Antimycin A was obtained from Serva, SHAM was from Aldrich, α-ketoglutarate was from Fluka Ab., DL-lactate was from UCB, and carbonyl cyanide *m*-chlorophenylhydrazone was from Calbiochem. All other materials were purchased from E. Merck Ab. Enzymes were obtained from Boehringer Mannheim, except α-amylase which was obtained from E. Merck Ab.

Substrates, nucleotides, and inhibitors were dissolved in glass-distilled water and neutralized when necessary. Antimycin A and SHAM were dissolved in *N,N* dimethylformamide. Oligomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were dissolved in ethanol.

RESULTS

Oxidase activities of M. tomentosa mitochondria. In Table 1 are given the different substrates which are oxidized by normal and EBr mitochondria. With respect to the oxidation rates it can be seen that with both types of mitochondria the highest activities were found with NADH and with succinate. Normal mitochondria respire best with NADH, EBr mitochondria with succinate. Although normal and EBr mitochondria contained a high activity of malate dehydrogenase (respectively 4,900 and 2,600 nmol of malate formed from *cis*-oxaloacetate per min and per mg of protein), no oxygen uptake in the presence of malate could be measured. Similarly, no oxygen uptake was found with α-ketoglutarate (plus malate) or with pyruvate (plus malate).

Effect of CN and SHAM on mitochondrial oxidase activities. Previous results for whole cells indicated that the oxygen uptake by normal cells was highly sensitive to CN and insensitive to SHAM, whereas opposite results were obtained with cells grown in the presence of drugs (10). Figure 1 and 2 indicate that the effects of CN and SHAM on the oxygen uptake by mitochondria isolated from normal and EBr

TABLE 1. *Oxidase activities of mitochondria from M. tomentosa*

Substrate	Oxidase activity[a] (natoms of O/min per mg of protein)	
	Normal mitochondria	EBr mitochondria
NADH	488	82
NADPH	33	19
Succinate	225	116
Lactate	11	18
Isocitrate	28	30

[a] Mitochondria from normal and EBr cells were suspended in respiration buffer containing 0.5 ADP, 8 mM NaH_2PO_4, and 5 mM $MgCl_2$ for the measurement of the oxidase activities.

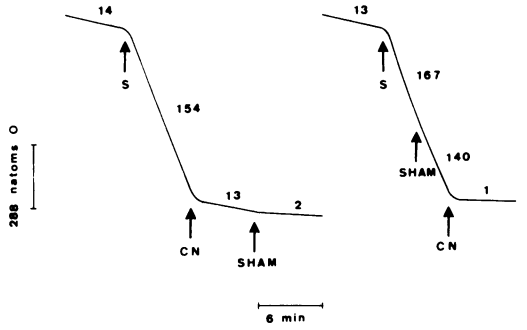

FIG. 1. *Effect of CN and SHAM on the succinate oxidase activity in normal mitochondria. Mitochondrial proteins (0.81 mg) were suspended in 3 ml of respiration buffer containing 0.5 mM ADP, 8 mM NaH_2PO_4, and 5 mM $MgCl_2$ for the polarographic measurements of oxygen consumption. The arrows indicate the subsequent additions of 20 mM succinate (S), 1 mM CN, or 400 μg of SHAM per ml. The figures above the traces represent respiratory rates expressed as nanoatoms of O/minute per milligram of protein.*

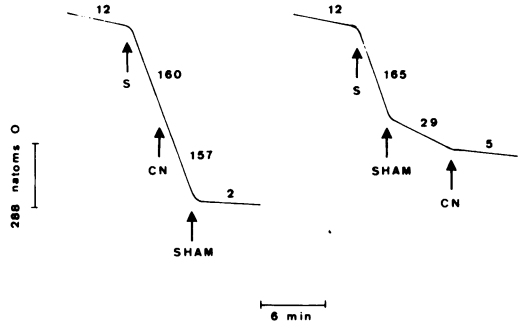

FIG. 2. *Effect of CN and SHAM on the succinate oxidase activity in EBr mitochondria. Mitochondrial proteins (0.84 mg) were suspended in 3 ml of respiration buffer containing 0.5 mM ADP, 8 mM NaH_2PO_4, and 5 mM $MgCl_2$ for the polarographic measurements of oxygen consumption. The arrows indicate the subsequent additions of 20 mM succinate (S), 1 mM CN or 400 μg of SHAM per ml. The figures above the traces represent respiratory rates expressed as nanoatoms of O/minute per milligram of protein.*

cells are similar to those observed for whole cells. The effects were found when either succinate or NADH, NADPH, or isocitrate were used as resipiratory substrates. The results indicate that these substrates are oxidized by the normal respiratory chain in mitochondria from normal cells and predominantly by the alternate oxidase in EBr mitochondria. The same results were obtained when antimycin A (0.5 nmol of protein per μg) was substituted for CN. The experiments provide evidence that the alternate oxidase is associated with the mitochondrial fraction of the cells.

Distinctly different results were obtained in the presence of lactate as substrate. In both types of mitochondria lactate oxidation was insensitive to antimycin A or SHAM but could be completely inhibited with CN.

Effect of ADP on the oxidase activities. Addition of ADP to state 4 mitochondria results in a shift to state 3 mitochondria and the ratio of state 3 respiration to the subsequent state 4 respiration (after exhaustion of the added ADP) is defined as the respiratory control ratio (3).

The addition of ADP to normal mitochondria of *M. tomentosa* respiring with NADH or succinate resulted in a nearly twofold increase of the respiratory rate (Table 2). However, the mitochondria did not return to state 4 and no respiratory control or ADP-O ratios could be calculated. The stimulated oxidase activities were sensitive to CN and insensitive to SHAM.

With EBr mitochondria respiring with NADH or succinate, the addition of ADP resulted in an even higher stimulation of the oxygen uptake but again no respiratory control or ADP-O ratios could be calculated. However, in this instance, the stimulated respiratory activity was mediated by the alternate oxidase as this respiration was insensitive to CN but sensitive to SHAM.

The lack of respiratory control in mitochondria of *M. tomentosa* could be due to the presence of an active ATPase. However, this seems unlikely for EBr mitochondria as no reports have appeared so far on the occurrence of an alternate oxidase-associated oxidative phosphorylation. The incubation mixture for the study of the effect of ADP in EBr mitochondria was then modified to obtain more data on the nature of the increased oxygen uptake in the presence of ADP. The results are given in Table 3. The omission of phosphate from the reaction mixture did not decrease the stimulating effect exerted by ADP. The omission of magnesium resulted in a 50% decrease of the stimulating effect of ADP but the stimulation was similar to this obtained without addition of both phosphate and magnesium. Thus no external phosphate or magnesium seem necessary to obtain an ADP effect, although external magnesium enhances the effect. Uncouplers such as 2,4 dinitrophenol (2,4 DNP) or CCCP were without effect. Similarly no decrease in the oxygen uptake in the presence of ADP was observed in the presence of atractyloside or oligomycin.

Effect of nucleotides on the CN-insensitive respiration. In the foregoing experiments it became clear that the ADP effect was not directly related to the process of oxidative phosphoryla-

TABLE 2. *Effect of ADP on the NADH oxidase and succinate oxidase activities in normal and EBr mitochondria*

Additions	Oxidase activity (natoms of O/min per mg of protein)[a]					
	Normal mitochondria			EBr mitochondria		
	No	+CN[b]	+SHAM[b]	No	+CN	+SHAM
NADH[b]	285	15	267	24	15	2
NADH + ADP[b]	414	17	395	95	92	1
Succinate[b]	158	13	145	22	18	2
Succinate + ADP	293	15	273	137	135	3

[a] The oxygen uptake was measured in respiration buffer containing 8 mM NaH_2PO_4 and 5 mM $MgCl_2$.
[b] Final concentrations were 0.5 mM NADH, 20 mM succinate, 0.2 mM ADP, 1 mM CN, and 400 μg of SHAM per ml.

TABLE 3. *Effect of medium composition, uncouplers, and inhibitors on the succinate oxidase activity in EBr mitochondria*

Additions	Succinate oxidase (natoms of O/min per mg of protein)	
	No ADP	Plus ADP
Series 1[a]		
None	25	82
$MgCl_2$	24	155
NaH_2PO_4	25	75
$MgCl_2$ + NaH_2PO_4	23	145
Series 2[a]		
2,4 DNP	27	160
CCCP	28	158
Atractyloside	24	148
Oligomycin	23	145
Antimycin A	20	141
CN	19	143
SHAM	5	5

[a] The first series of experiments (1) were performed in respiration buffer, the second series (2) in respiration buffer containing 8 mM NaH_2PO_4 and 5 mM $MgCl_2$. Final concentrations were 10 mM succinate, 0.5 mM ADP, 5 mM $MgCl_2$, 8 mM NaH_2PO_4, 0.3 mM 2,4 DNP, 1.5 μM CCCP, 1 mM CN, and 400 μg of SHAM. Atractyloside, oligomycin, and antimycin A were added to final concentrations of 0.25, 0.5, and 0.5 nmol/mg of protein, respectively.

tion. This was confirmed by the results given in Table 4 where it is shown that other nucleotides as well are effective in stimulating the oxygen uptake by EBr mitochondria. Adenosine 5'-monophosphate (AMP), ATP, and guanosine 5'-monophosphate (GMP) but not uridine 5'-monophosphate (UMP) or cytidine 5'-monophosphate (CMP) show high stimulatory effects on the oxidation of succinate by EBr mitochondria.

Actually it was found that AMP rather than ADP at ATP was responsible for the stimulation. In Fig. 3 it can be seen that the addition of AMP immediately induces the stimulatory effect, while the effect with ADP or ATP reaches a maximum only after the addition of magnesium. Moreover, it was shown by electrophoretic analysis of the reaction mixtures that EBr mitochondria effect a rapid transformation of ATP and ADP to AMP in the presence of magnesium. ADP can be converted to AMP by the action of myokinase and ATP can be converted to AMP by the combined action of myokinase and ATPase. Both activities are abundantly present in EBr mitochondria (Table 5) and are known to require magnesium.

In Table 4 it can be seen that AMP stimulates the oxidase activity but that neither adenosine 2'-monophosphate (2'-AMP) nor adenosine 3'-monophosphate (3'-AMP) has any effect on the reaction.

The stimulation by AMP of the oxygen uptake in EBr mitochondria is not restricted to the oxidation of succinate. NADH, NADPH, and isocitrate also showed a higher oxidation rate in the presence of AMP. In all cases the

TABLE 4. *Effect of nucleotides on the succinate oxidase activity in EBr mitochondria*

Nucleotide[a]	Succinate oxidase[b] (natoms of O/min per mg of protein)
None	3
ATP	71
ADP	73
AMP	78
CMP	3
GMP	52
UMP	3
2'-AMP	3
3'-AMP	3

[a] Final concentrations of the nucleotides in the reaction mixture were 0.95 mM ATP, 1 mM ADP, 0.7 mM AMP, 0.65 mM CMP, 1 mM GMP, 0.8 mM UMP, 0.6 mM 2'-AMP, and 0.7 mM 3'-AMP.
[b] The oxygen uptake was measured in respiration buffer containing 5 mM $MgCl_2$ and 10 mM succinate.

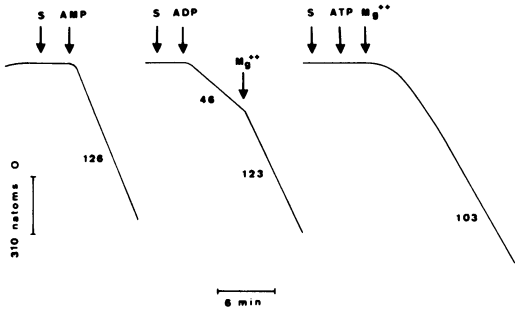

FIG. 3. *Polarographic traces showing the effect of AMP, ADP, and ATP on the oxidation of succinate by EBr mitochondria. Mitochondrial proteins (0.93 μg) were incubated in 3 ml of respiration buffer containing 1 mM CN. At the points indicated additions were made of 10 mM succinate (S), 2.5 mM $MgCl_2$, 0.25 mM AMP, 0.46 mM ADP, or 0.58 mM ATP. The figures below the traces give oxygen consumption rates in nanoatoms of O/minute per milligram of protein.*

TABLE 5. *ATPase and myokinase activity in normal and EBr mitochondria*

Activity	Normal mitochondria	EBr mitochondria
ATPase[a]	1,396	881
Myokinase[b]	1,060	804

[a] Expressed as nanomoles of ATP hydrolyzed per minute per milligram of protein.

[b] Expressed as nanomoles of ATP formed per minute per milligram of protein.

stimulation was inhibited by SHAM but not by CN. When lactate was used as a respiratory substrate there was no effect of AMP on the oxidation rate. Similarly, no stimulation by AMP was found on the oxidase activities in normal mitochondria.

Kinetic parameters of the stimulation by AMP and the inhibition with SHAM of the alternate oxidase. Double-reciprocal plots of the AMP-stimulated succinate oxidase activity versus the concentration of AMP or succinate were obtained by varying the concentration of each of these compounds at a fixed concentration of the other. The plots permit calculation of the concentration of AMP or succinate which gives half the maximal velocity under the experimental conditions used. An apparent K_m of 23 μM for AMP and of 0.6 mM for succinate were found (Fig. 4 and 5). Similar determinations made in the presence of SHAM indicate that the inhibition exerted by this compound is noncompetitive with respect to both AMP and succinate as can be deduced from the intercepts on the abscissae.

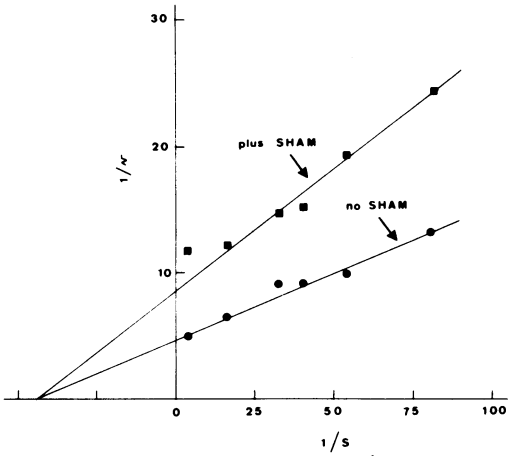

FIG. 4. *Double-reciprocal plot of v (microatoms of O/minute per milligram of protein) and S (mM AMP) for the determination of the Michaelis-Menten constant for AMP of the succinate oxidase activity in EBr mitochondria. Succinate oxidase activity was measured polarographically with 0.98 mg of mitochondrial proteins in the presence of 1 mM CN and 10 mM succinate. AMP was added to final concentrations between 0.01 and 0.25 mM and SHAM to a final concentration of 20 μg/ml. The apparent K_m for AMP is 23 μM as determined from the intercept on the 1/S axis.*

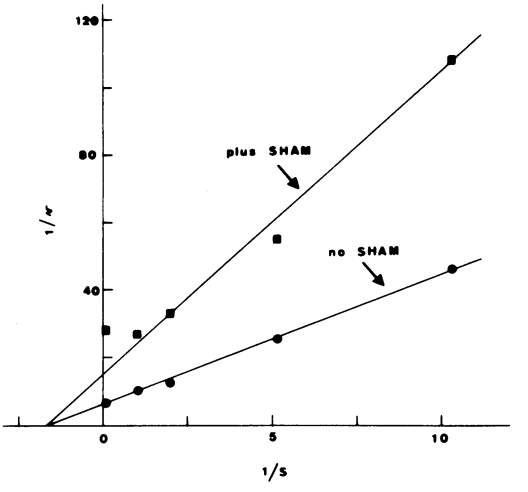

FIG. 5. *Double-reciprocal plot of v (microatoms of O per minute per milligram of protein) and S (mM succinate) for the determination of the Michaelis-Menten constant for succinate of the AMP-stimulated succinate oxidase activity in EBr mitochondria. Succinate oxidase activity was measured polarographically with 0.98 mg of mitochondrial proteins in the presence of 1 mM CN and 0.25 mM AMP. Succinate was added to final concentrations between 0.1 and 10 mM. The final concentration of SHAM was 20 μg/ml. The apparent K_m for succinate is 0.6 mM as determined from the intercept on the 1/S axis.*

The sensitivity of the AMP-stimulated alternate oxidase to SHAM was measured by adding various amounts of this inhibitor to mitochondria respiring with succinate in the presence of CN. The half-maximal inhibition of the respiration, as determined from a Dixon plot of the titration data, is reached at a concentration of 20 μg/ml of SHAM (0.13 mM) (Fig. 6). This is within the range of values reported for the sensitivity towards SHAM of the CN-insensitive respiration in *poky* and higher plant mitochondria (13, 16, 23).

DISCUSSION

Mitochondria of *M. tomentosa* were obtained after disruption of the cells with a French press. Less drastic methods involving the preparation of spheroplasts by digestion of the cell walls with snail gut enzymes (8) in combination with or without cellulase (18) or Pronase (15) were unsuccessful. Mechanical breakage of the cells is a rather severe method and this might be responsible for the lack of mitochondrial oxidase activity with malate, α-ketoglutarate, or pyruvate.

Good yields of mitochondria were only obtained after incubation of the crude extracts with α-amylase. When this treatment was omitted, most of the oxidase activities were found in the high-speed supernatants of the extracts. Probably α-amylase hydrolyzes a polysaccharide that hinders the sedimentation of the mitochondria. Such a polysaccharide may have resulted from the high concentrations of glucose used in the growth medium of the osmotolerant microorganism. We found that no α-amylase treatment was necessary for the preparation of mitochondria from ethanol-grown cells.

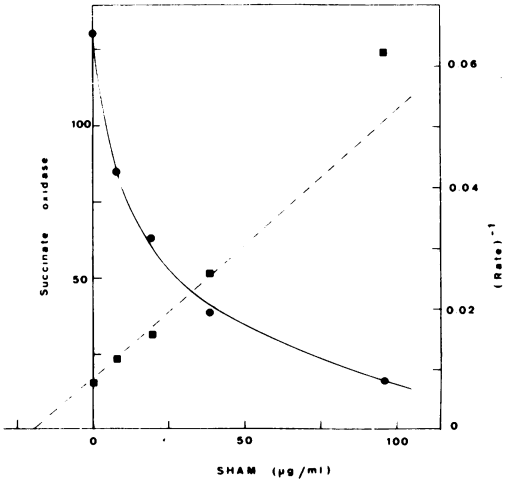

FIG. 6. *Titration with SHAM of the AMP-stimulated succinate oxidase activity in EBr mitochondria. Succinate oxidase activity (nanoatoms of O/minute per milligram of protein) was measured polarographically with 0.9 mitochondrial proteins in the presence of 1 mM CN, 0.25 mM AMP, and 10 mM succinate. SHAM was added to final concentrations between 0 and 100 μg/ml. The dashed line represents a Dixon plot of the titration data.*

The oxidation of NADH, NADPH, succinate, and isocitrate is insensitive to SHAM but sensitive to CN when mitochondria are isolated from cells grown in the absence of EBr. With mitochondria isolated from cells grown in the presence of EBr, the oxidations are greatly sensitive to SHAM but insensitive to CN. Similar effects were also observed with whole cells (10). The present results imply that these activities are localized in the mitochondria and that normal mitochondria use the normal cytochrome system, whereas EBr mitochondria predominantly use the alternate oxidase.

Experiments with whole cells (10) indicated that the respiration in normal cells of *M. tomentosa* could be mediated by a branched respiratory system similar to the one found in *poky* (12). The present results do not allow extention of this observation to the mitochondria. This might be due to the growth conditions of the cells. It is very likely that normal mitochondria may shift part of their respiration from the normal chain to the alternate oxidase as (Hanssens and Verachtert, in preparation) the activity of the alternate oxidase is very low in normal cells taken from the exponential growth phase, whereas it can mediate about 50% of the total respiration in cells from the stationary phase of growth. In the present work we have prepared mitochondria from exponential growth-phase cells. Consequently we found that the respiration in these mitochondria is exclusively mediated by the normal cytochrome chain and it will probably be necessary to isolate mitochondria from stationary-phase cells to observe the branched respiratory system in mitochondria of *M. tomentosa*.

Mitochondria of cells grown in the presence of EBr showed almost no oxidase activity unless AMP was added. The stimulation by AMP is specifically associated with the alternate oxidase since it was not inhibited by CN or antimycin A but could be completely blocked by SHAM. ADP, or ATP in the presence of magnesium and GMP were also found to stimulate the respiration in these mitochondria. The mode of action of AMP in stimulating the alternate oxidase is not known. Possibly it might be an allosteric effector. A stimulation by AMP of a novel oxidase activity in mitochondria of *Eu-*

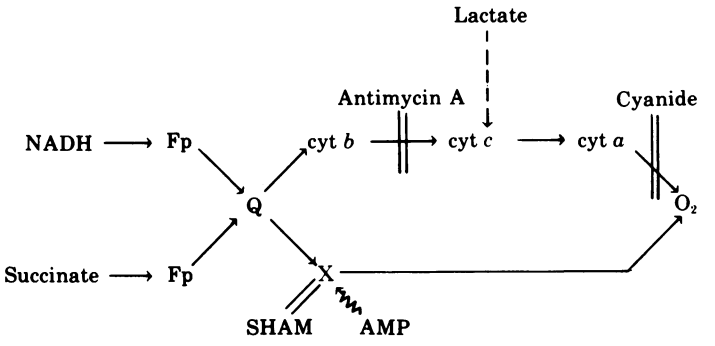

FIG. 7. *Scheme of electron transport in M. tomentosa. The double lines represent the sites of inhibition, the zig-zag arrow represents the stimulation by AMP.*

glena gracilis grown on ethanol or on succinate plus antimycin A has been reported by Sharpless and Butow (17). This novel oxidase resembles the alternate oxidase in EBr mitochondria of *M. tomentosa* in that it was insensitive to CN or antimycin A. However, there were also some differences. AMP stimulates the oxidation of lactate in *E. gracilis* but has no effect on this activity in mitochondria of *M. tomentosa*. Moreover, ethanol-grown cells of *M. tomentosa* contain only the normal respiratory chain.

The stimulatory effect of ADP on the oxygen uptake by normal mitochondria is different from the effect of nucleotides in EBr mitochondria as the oxygen uptake is not affected by AMP or SHAM, but is completely inhibited by CN. The effect of ADP on normal mitochondria might be explained as resulting from oxidative phosphorylation. The lack of respiratory control as measured polarographically could be due to the high ATPase activity in the mitochondria. Another method for determining oxidative phosphorylation was attempted by measuring the synthesis of glucose 6-phosphate and the disappearance of inorganic phosphate after incubation of the mitochondria with ADP, inorganic phosphate, $MgCl_2$, glucose, and hexokinase (19). It was found that a rapid synthesis of gucose 6-phosphate occured but that no inorganic phosphate was consumed. It might be that ATP, necessary for the synthesis of glucose 6-phosphate, has been formed through the action of myokinase on ADP. The preparation of mitocondria with low myokinase activity will be necessary for further work on this subject.

The results obtained in this work together with previous results (10) allow a presentation of the following scheme of electron transport in *M. tomentosa* (Fig. 7). Because one system (present in normal mitochondria) is sensitive to CN or antimycin A and the other system (present in EBr mitochondria) is sensitive to SHAM, the branch point must be localized before the inhibitory sites of these compounds and is tentatively presented by coenzyme Q. The stimulation by AMP is restricted to one branch. The oxidation of NADH and succinate can occur via both branches but the oxidation of lactate must be restricted to one branch as it is completely sensitive to CN, but is neither inhibited by SHAM nor stimulated by AMP in both types of mitochondria. Lactate probably provides electrons at the level of cytochrome *c* because its oxidation is insensitive to antimycin A.

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