Characterization of Cyanide-Insensitive Respiration in Mitochondria and Submitochondrial Particles of *Moniliella tomentosa*

By Jos VANDERLEYDEN, Jochen KURTH* and Hubert VERACHTERT
Laboratory for Industrial Microbiology and Biochemistry, University of Leuven, 3030
Heverlee-Louvain, Belgium

(Received 28 December 1978)

Mitochondria and submitochondrial particles of the osmophilic yeast-like fungus *Moniliella tomentosa* may respire by means of two pathways: a normal cytochrome pathway, sensitive to cyanide and antimycin A, and an alternative pathway, which is insensitive to these inhibitors but is specifically inhibited by salicylhydroxamic acid. The affinities of both oxidases for succinate and NADH as substrates, for O₂ as terminal electron acceptor, and for AMP as stimulator of the alternative oxidase were determined. 1. Submitochondrial particles of *M. tomentosa* may also respire by means of a cyanide-sensitive and/or cyanide-insensitive system. 2. The activities of both oxidases as compared with the total activity are roughly the same in submitochondrial particles as in the original mitochondria. 3. The terminal oxidase of the cyanide-insensitive pathway requires a 10-fold higher O₂ concentration for saturation than does cytochrome *c* oxidase. 4. The apparent K_m for succinate is about 3 times higher for the alternative than for the normal oxidase when measured in mitochondria, and 4–10 times higher when measured in submitochondrial particles. The apparent K_m for NADH is roughly the same for both oxidases. 5. The apparent K_m values of both oxidases for succinate are always lower in submitochondrial particles than in mitochondria. 6. The apparent K_m for AMP, acting as a stimulator of the alternative oxidase, is the same (25 μ M) in mitochondria as in submitochondrial particles. These results are discussed in the light of the structure and localization of the components of the alternative oxidase.

Mitochondrial cyanide-insensitive respiration is known to take place in a number of higher plants and eukaryotic micro-organisms under certain physiological conditions (Henry & Nyns, 1975). The nature of this alternative oxidase and the branchpoint from the main cytochrome chain still remain obscure. The substituted hydroxamates that are inhibitors of this alternative pathway (Schonbaum *et al.*, 1971) were considered to inhibit by chelation of iron-sulphur centres (Bendall & Bonner, 1971; Lyr & Schewe, 1975; Henry *et al.*, 1977; Lance *et al.*, 1978). E.p.r., however, revealed that hydroxamates may also interact with ubiquinone species (Rich & Bonner, 1977; Bonner & Rich, 1978). Moore & Rupp (1978) in mung-bean mitochondria observed a specific interaction of salicylhydroxamic acid with semiquinones. This strongly supports the scheme of Rich & Moore (1976), which integrates the alternative oxidase of plant mitochondria into the proton-motive ubiquinone cycle proposed by Mitchell (1975*a,b*) for mammalian mitochondria.

In previous studies we have pointed out that the fungus *Moniliella tomentosa* may respire by means

of two mitochondrial oxidase systems (Hanssens *et al.*, 1974). The activities of both oxidase systems, relative to the total respiratory flux, are related to the growth stage and the culture conditions. Normal cells in the exponential growth phase respire exclusively by the normal chain, whereas cells grown on chloramphenicol- or ethidium bromide-containing media respire mainly by the alternative oxidase (Hanssens & Verachtert, 1976). Cells in the stationary phase always made more use of the alternative oxidase. Cells grown in the presence of propan-1-ol use both pathways almost equally in the late exponential growth phase (Vanderleyden *et al.*, 1978). Furthermore the alternative oxidase in mitochondria from *M. tomentosa* is characterized by its stimulation by AMP (Hanssens & Verachtert, 1976). Various workers have established that the development of the cyanide-insensitive respiration in potato tuber slices could be related to modifications of the mitochondrial membrane (Nakamura & Asahi, 1976; Waring & Laties, 1977*a,b*). Therefore we decided to study the localization of the alternative oxidase. This paper reports the presence of the alternative oxidase in submitochondrial particles, and some kinetic results for both the normal and the alternative oxidase in mitochondria and submitochondrial particles from *M. tomentosa*.

* Present address: Sektion Biowissenschaften, Bereich Biochemie, Karl Marx Universität Leipzig, 701 Leipzig, German Democratic Republic.

Experimental

Preparations

Micro-organism and growth of cells. The organism used was the osmophilic obligate aerobic yeast-like fungus *Moniliella tomentosa*, CBS 461.67. Cells were grown in 100ml of medium in 500ml Erlenmeyer flasks at 30°C on a reciprocal shaker. The standard medium for 'normal' cells consisted of 10% glucose, 1% Oxoid yeast extract and 0.1% urea (GYU medium). The standard medium was supplemented with 4mg of chloramphenicol/ml for 'CAP' cells and 0.6% (w/v) propan-1-ol for 'propanol' cells. 'Normal' cells were grown for 19h, 'CAP' cells for 29h and 'propanol' cells for 36h.

Preparation of mitochondria. Mitochondria were prepared in an Aminco French pressure cell as described by Hanssens & Verachtert (1976) with the following modifications. The cells were suspended in a medium consisting of 0.8M-sucrose, 20mM-EDTA and 0.1% (w/v) bovine serum albumin, adjusted to pH7.2. The extract was carefully kept at pH6.8 by addition of 1M-NaOH. Isolated mitochondria were transferred to liquid N₂ until required. Mitochondria used for the determination of K_m values for AMP were prepared by the original procedure (Hanssens & Verachtert, 1976), as only this procedure allowed us to obtain mitochondria that showed an absolute requirement for AMP.

Preparation of submitochondrial particles. The submitochondrial particles were prepared by treating the mitochondria with a French press at 55.2MPa, as described by Wilson & Bonner (1970). After disruption of the mitochondria, the suspension was centrifuged at 12000g for 20min in the SS34 rotor of a Sorvall centrifuge to remove remaining intact mitochondria. The submitochondrial particles were spun down at 100000g for 60min in a Beckman L5-65 ultracentrifuge. The pellet was washed and centrifuged once more at 100000g. The submitochondrial particles were stored in liquid N₂ until required.

Analytical procedures

O₂ consumption. O₂ consumption was measured polarographically at 25°C with a Clark-type oxygen electrode (Yellow Springs Instrument Co.) inserted into a water-jacketed cuvette which was on a magnetic stirrer. Cell respiration was measured in 3ml of 3,3-dimethylglutaric acid/NaOH buffer (20mM, pH5.8), containing 1% (w/v) glucose; 3–4mg dry wt. of cells was used. O₂ uptake by mitochondria and submitochondrial particles was measured in sucrose buffer [0.8M-sucrose, 5mM-Tris/HCl, 5mM-KCl, 5mM-MgCl₂, 20mM-KH₂PO₄, 0.3% (w/v) bovine serum albumin, pH6.8], with the use of 0.4–0.8mg of mitochondrial protein or 0.7–1.5mg of submito-

chondrial particle protein. O₂ uptake is expressed as ng-atom of O/min per mg of protein.

Determination of K_m values. The K_m values for succinate and NADH were determined from the rate of O₂ consumption after small additions of substrate to the reaction mixture given above. The concentration of NADH in the solutions was measured spectrophotometrically by using a millimolar absorption coefficient of 6.22 litre·mmol⁻¹·cm⁻¹ at 340nm.

Respiratory rates at various O₂ concentrations were determined from slopes drawn at a tangent to points along curves made with a linear recorder connected to the polarograph. Accuracy of the method was increased by expanding the sensitivity of the instrument so that the normal chart span was used to record respiratory rates at O₂ concentrations between 10 and 0% saturation. The reciprocal of velocity was plotted against the reciprocal of O₂ concentration, and the O₂ concentration supporting half-maximal respiratory rate (K_m) was calculated.

The K_m for AMP was determined from the rate of O₂ consumption after successive small additions of AMP to the reaction mixture in the presence of 1mM-KCN, 20mM-succinate or 1mM-NADH. The concentration of AMP in the solutions was measured spectrophotometrically by using a millimolar absorption coefficient of 15.3 litre·mmol⁻¹·cm⁻¹ at 260nm.

Protein determination. Mitochondrial and submitochondrial particle protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Proteins were solubilized with 0.2% (w/v) deoxycholate before determination.

Materials. All biochemicals were obtained from Sigma Chemical Co. All chemicals used were of analytical grade.

Results

Comparison of oxidase activities in mitochondria and submitochondrial particles of *M. tomentosa*

Table 1 compares the NADH oxidase and succinate oxidase activities of the normal and the alternative pathway in mitochondria and submitochondrial particles. In accordance with earlier reports (Hanssens & Verachtert, 1976; Vanderleyden *et al.*, 1978), mitochondria from 'normal' and 'CAP' cells possess either the normal or the alternative oxidase, whereas both oxidases are present in mitochondria from 'propanol' cells. The oxidase activities in the corresponding submitochondrial particles indicate that the normal oxidase (measured in the presence of salicylhydroxamic acid) as well as the alternative oxidase (measured in the presence of CN+AMP) are well retained in submitochondrial particles. Furthermore the results obtained for the oxidase

Table 1. *NADH oxidase and succinate oxidase activities of mitochondria and submitochondrial particles from 'normal', 'propanol' and 'CAP' cells of M. tomentosa*

The oxidase activity was measured polarographically with either 1 mM-NADH or 20 mM-succinate as substrate. The reaction mixture (final volume 3 ml, temperature 25°C, pH 6.8) consisted of 0.8 M-sucrose, 5 mM-Tris/HCl, 5 mM-KCl, 5 mM-MgCl₂, 20 mM-KH₂PO₄, 0.3% (w/v) bovine serum albumin and mitochondria (0.4–0.8 mg of protein) or submitochondrial particles (0.7–1.5 mg of protein). The activity of the normal oxidase was measured in the presence of 2.6 mM-salicylhydroxamic acid, and the activity of the alternative oxidase was measured in the presence of 1 mM-KCN and 1 mM-AMP. Abbreviation: SHAM, salicylhydroxamic acid.

Additions	Oxidase activity (ng-atoms of O/min per mg of protein)					
	'Normal'		'Propanol'		'CAP'	
	Mitochondria	Submitochondrial particles	Mitochondria	Submitochondrial particles	Mitochondria	Submitochondrial particles
NADH	456	286	486	210	197	51
NADH+SHAM	440	286	328	165	15	4
NADH+KCN	5	12	285	23	187	49
NADH+KCN+AMP	5	12	290	170	187	184
NADH+KCN+AMP+SHAM	5	0	5	0	4	0
Succinate	225	134	327	122	129	27
Succinate+SHAM	225	134	210	87	10	5
Succinate+KCN	10	5	190	19	119	22
Succinate+KCN+AMP	10	5	210	89	137	134
Succinate+KCN+AMP+SHAM	4	0	0	0	5	0

Fig.1. *Effect of AMP on the alternative oxidase in mitochondria and submitochondrial particles from 'propanol' and 'CAP' cells of M. tomentosa*

Succinate oxidase activity of mitochondria (a) and submitochondrial particles (b) was measured polarographically as indicated in Table 1. Mito and SMP indicate addition of mitochondria and submitochondrial particles respectively. At the points indicated additions were made of 20 mM-succinate (Suc), 1 mM-KCN (CN), 1 mM-AMP and 2.6 mM-salicylhydroxamic acid (SHAM). The values above the traces are rates of O₂ consumption in ng-atoms of O/min per mg of protein.

Fig. 2. Polarographic traces of O_2 uptake as a function of time in 'propanol' cells of *M. tomentosa*

The reaction mixture (final volume 3 ml; temperature 25°C ; pH 5.8) consisted of 20 mM-3,3-dimethylglutaric acid/NaOH, 1% (w/v) glucose and $235 \mu\text{M}-O_2$. The reaction was started by addition of cell suspension, corresponding to 3 mg dry wt. of cells. ●, 2.6 mM-Salicylhydroxamic acid; ▲, 1 mM-KCN.

activities in mitochondria and submitochondrial particles of 'propanol' cells demonstrate that both oxidases in submitochondrial particles function with the same activities (relative to the total oxidase activity, measured in the absence of any inhibitor) as in the intact mitochondria. It should be noted, however, that the effect of AMP on the alternative oxidase is quite different when measured with mitochondria and submitochondrial particles. Hanssens & Verachtert (1976) reported that, unless AMP was added to the reaction mixture, only a small activity of the alternative oxidase could be measured. However, with mitochondria prepared by the modified procedure (as indicated in the Experimental section) the alternative oxidase, measured in the presence of CN, always operated at full capacity whether or not exogenous AMP was added to the reaction mixture (Fig. 1a). When these mitochondria were disrupted to produce submitochondrial particles, the alternative oxidase again needed exogenous AMP, as shown in Fig. 1(b). Preliminary experiments have indicated that the different behaviour towards exogenous AMP can be attributed to the different concentrations of adenine nucleotides in the mitochondrial preparations.

O_2 affinity of the terminal oxidases of both electron-transport pathways

O_2 consumption as a function of O_2 concentration for both respiratory pathways was estimated for

whole cells, mitochondria and submitochondrial particles. Fig. 2 shows the O_2 consumption of 'propanol' cells through the cytochrome pathway (+salicylhydroxamic acid) and the alternative pathway (+CN) as a function of time. O_2 consumption in the presence of salicylhydroxamic acid remains linear down to very low concentrations of O_2 ($8 \mu\text{M}-O_2$), pointing to an oxidase with a high affinity for O_2 and characteristic of cytochrome *c* oxidase (Chance, 1965). In the presence of CN, the O_2 uptake slows down at about $45 \mu\text{M}-O_2$, suggesting the participation of an oxidase with a lower affinity for O_2 than the cytochrome *c* oxidase has. In fact, a mean K_m value of $12 \mu\text{M}-O_2$ could be calculated from the O_2 -consumption curve in the presence of CN. Calculations in the presence of salicylhydroxamic acid were not possible from this graph.

The O_2 molarity values of K_m for both oxidases in the different types of mitochondria are presented in Table 2. The results (especially with mitochondria from 'propanol' cells) indicate that the alternative oxidase needed almost a 10-fold higher O_2 concentration than did the normal oxidase, to reach the half-maximal respiratory rates. The results in Table 2 also demonstrate that the kinetics of both oxidases with respect to their affinity for O_2 were not altered whether present alone or in combination. The K_m values for submitochondrial particles are in complete accordance with those for intact mitochondria (Table 3). No determinations were made with submitochondrial particles from 'normal' cells.

The absolute values given in Tables 2 and 3 also establish that the observed difference between the two oxidases with respect to their affinity for O_2 is due to the different natures of the two terminal oxidases and not to different accessibility of O_2 towards the oxidases. In the latter case there would have been at least a difference between mitochondria and submitochondrial particles.

Table 2. O_2 concentration supporting half-maximal respiratory rate (K_m) in *M. tomentosa* mitochondria. The K_m was determined from the rate of O_2 consumption at different concentrations of O_2 in solution. Oxidase activity was measured polarographically under the conditions described in Table 1.

Source of mitochondria	Substrate	O_2 concentration (μM)	
		Normal oxidase	Alternative oxidase
'Normal' cells	NADH	<3	Not present
	Succinate	<3	
'Propanol' cells	NADH	<3	8
	Succinate	<3	11
'CAP' cells	NADH	Not present	11
	Succinate		12

Kinetic parameters for the oxidation of NADH and succinate through the normal and the alternative pathway

Sharpless & Butow (1970) proposed that the activity of the AMP-stimulated oxidase in *Euglena gracilis* mitochondria could be fulfilled by components of the succinate dehydrogenase. The K_m of succinate dehydrogenase for succinate with phenazine methosulphate as electron acceptor differed by more than a factor of 10 between the two oxidases. It was also observed that the alternative oxidase in some organisms showed a higher activity with succinate than with other substrates (Sharpless & Butow, 1970; Wedding *et al.*, 1973; Van Der Plas, 1977; Edwards & Lloyd, 1978b). Table 4 summarizes the K_m values for succinate for both pathways. The apparent K_m values for succinate differ by a factor of 3 between the two oxidases. The same absolute values with each of the two oxidases were obtained whether present alone (mitochondria from 'normal' and 'CAP' cells) or in combination (mitochondria from 'propanol' cells). These results indicate that a second succinate dehydrogenase is involved in the alternative pathway or that some components of the same succinate-coenzyme Q

(ubiquinone) oxidoreductase complex are altered when the normal or the alternative oxidase is active.

The same experiments were performed with submitochondrial particles (Table 5). With respect to the affinity for succinate, a difference of a factor of 10 existed between the succinate dehydrogenase operating in submitochondrial particles from 'normal' cells (normal oxidase) and the dehydrogenase operating in submitochondrial particles from 'CAP' cells (alternative oxidase). In submitochondrial particles from 'propanol' cells only a difference of a factor of 4 between the two succinate dehydrogenases was observed. These results reveal that the kinetics of the alternative oxidase with respect to its affinity for succinate are altered whether the alternative oxidase is present alone or in combination with the normal oxidase. However, this is only observed in submitochondrial particles.

Furthermore it can be seen that there was no difference between the two oxidases with respect to their kinetics of NADH oxidation.

Kinetic parameter for the stimulation of the alternative oxidase by AMP

The activity of the alternative oxidase in *M. tomentosa* can be markedly stimulated by AMP as observed by Hanssens & Verachtert (1976). Table 6 includes the K_m values for AMP of mitochondria and submitochondrial particles from *M. tomentosa*. Mitochondria were isolated by the original procedure described by Hanssens & Verachtert (1976). K_m values were determined from the rate of O_2 consumption after successive small additions of AMP to the reaction mixture in the presence of CN. Substrates were present in saturating concentrations. It is quite remarkable to observe that the same apparent K_m for AMP of the alternative oxidase was found in mitochondria as in submitochondrial particles. As

Table 3. O_2 concentration supporting half-maximal respiratory rate (K_m) in submitochondrial particles from *M. tomentosa*

The K_m was determined from the rate of O_2 consumption at different concentrations of O_2 in solution. Oxidase activity was measured polarographically under the conditions described in Table 1.

Source of submitochondrial particles	Substrate	O_2 concentration (μM)	
		Normal oxidase	Alternative oxidase
'Propanol' cells	NADH	<3	11
	Succinate	<3	12
'CAP' cells	NADH	Not present	12
	Succinate	Not present	11

Table 4. Kinetic parameter for the oxidation of succinate in *M. tomentosa* mitochondria

The K_m for succinate was determined from the rate of O_2 consumption after small additions of succinate to the reaction mixture given in Table 1. Respiratory rates were measured at saturating O_2 concentration.

Source of mitochondria	K_m (mM-succinate)	
	Normal oxidase	Alternative oxidase
'Normal' cells	2.7	Not present
'Propanol' cells	2.8	8.1
'CAP' cells	Not present	7.9

Table 5. Kinetic parameter for the oxidation of NADH and succinate in submitochondrial particles from *M. tomentosa*

The K_m values for NADH and succinate were determined from the rates of O_2 consumption after small additions of the substrate to the reaction mixture given in Table 1. Respiratory rates were measured at saturating O_2 concentration.

Source of submitochondrial particles	Substrate	K_m (mM-substrate)	
		Normal oxidase	Alternative oxidase
'Normal' cells	NADH	0.061	Not present
'Propanol' cells	NADH	0.039	0.043
'CAP' cells	NADH	Not present	0.045
'Normal' cells	Succinate	0.26	Not present
'Propanol' cells	Succinate	0.25	1.08
'CAP' cells	Succinate	Not present	2.43

Table 6. Kinetic parameter for the stimulation of the alternative oxidase by AMP in mitochondria and sub-mitochondrial particles from *M. tomentosa*

The K_m for AMP was determined from the rate of O_2 consumption after successive small additions of AMP to the reaction mixture given in Table 1. Respiratory rates were measured at saturating concentrations of O_2 and oxidation substrate. Mitochondria were isolated by the original procedure of Hanssens & Verachtert (1976).

Oxidation substrate		K_m (mM-AMP)	
		Mitochondria	Submitochondrial particles
'Propanol' cells	NADH	0.024	0.021
	Succinate	0.024	0.037
'CAP' cells	NADH	0.028	0.025
	Succinate	0.023	0.026

will be discussed below, this finding could give some indication of the site of action of AMP.

Discussion

From the data presented in this paper some conclusions can be drawn and discussed in the light of the previously described results and proposed hypotheses on cyanide-insensitive respiration.

Our experiments with submitochondrial particles (Table 1) clearly establish that all the components involved in the alternative oxidase of *M. tomentosa* are associated with and firmly bound to the inner mitochondrial membrane. However, our data also indicate that the activities of the normal and the alternative pathway are best conserved in those particles in which only one oxidase is expressed. When both oxidases are present, as in submitochondrial particles from 'propanol' cells, there is a considerable loss of activity, although both systems are preserved in the same proportions as in the corresponding mitochondria. This might indicate the necessity for a common compound that is easily lost in these mitochondria. This, as is suggested by comparing mitochondria from 'normal' and 'CAP' cells, might be ascribed to the specific growth conditions of the cells. Culture of *M. tomentosa* in the presence of propan-1-ol could, besides inducing the alternative pathway, give rise to some other modifications in the inner mitochondrial membrane.

Our data on O_2 affinity demonstrate that the terminal oxidase of the cyanide-insensitive pathway differs markedly from cytochrome *c* oxidase. A K_m value of $12 \mu M-O_2$ for the alternative oxidase in whole cells of *M. tomentosa* is close to the values obtained by other workers with cells of *Saccharomycopsis lipolytica* [K_m of $11.5 \mu M-O_2$ (Henry, 1976)], *Rhodotorula glutinis* [K_m of $23 \mu M-O_2$ (Matsunaki & Conti,

1966)], *Ustilago maydis* and *Neurospora sitophila* [K_m of $11-14 \mu M-O_2$ (Sherald & Sisler, 1970)]. These values are about 5–10 times higher than the K_m values of the normal oxidase of these cells. In our experiments $11 \mu M-O_2$ and less than $3 \mu M-O_2$ were recorded as mean K_m values in mitochondria for the alternative and the normal oxidase respectively (Table 2).

In isolated mitochondria from *Euglena gracilis*, Sharpless & Butow (1970) measured a mean K_m of $3.7 \mu M-O_2$ for the AMP-stimulated cyanide-insensitive oxidase and compared this value with the K_m value for the α -type cytochrome oxidases (K_m of $0.02-0.5 \mu M-O_2$) measured by Chance (1965). Although these values seem to indicate a larger difference between the two oxidases than is found in mitochondria of *M. tomentosa*, our data obtained with mitochondria and submitochondrial particles show a difference of a factor of 10 between the two oxidases with respect to their affinity for O_2 .

Hanssens *et al.* (1978) have proved that coenzyme Q is involved in the alternative oxidase of *M. tomentosa*. The values of the kinetic parameters for the oxidation of NADH and succinate (Tables 4 and 5) may suggest some modifications in the succinate-coenzyme Q oxidoreductase complex, when operating the alternative pathway, or the existence of a new complex for the alternative pathway. Moore & Rupp (1978) suggested from e.p.r. studies that the most likely candidates, at present, for the alternative oxidation mechanism are quinones in a hydrophobic environment. Since a quinone-binding protein that can serve as an electron carrier from succinate to ubiquinone has been described (Yu *et al.*, 1977), our kinetic analysis calls for preparative protein experiments with the succinate-coenzyme Q oxidoreductase complex. In submitochondrial particles, the observed K_m values for succinate in the main or the alternative pathway were always lower than in mitochondria. This could be explained by refusion of broken membranes and formation of inside-out particles as observed with Keilin-Hartree particles from heart muscle (Keilin & King, 1958). As succinate dehydrogenase is located on the matrix side of the inner membrane (De Pierre & Ernster, 1977), it might become more accessible after membrane inversion. In particles obtained by osmotic shock, refusion of broken membranes must occur (Wilson & Bonner, 1970). The results obtained with AMP do suggest nevertheless that membrane inversion or fusion does not occur under our experimental conditions. The K_m value is indeed the same with submitochondrial particles as with mitochondria and the inner mitochondrial membrane is not permeable to AMP (Munn, 1974). This also strongly suggests that AMP acts on a compound located on the outer surface of the inner membrane. If membrane inversion had occurred, some differences in the K_m value for AMP should have been observed with

submitochondrial particles compared with mitochondria. As the stimulation of the alternative oxidase by AMP is not restricted to one substrate, the compound on which AMP acts cannot be part of the dehydrogenases. Various workers (Edwards & Lloyd, 1978a; De Troostenbergh, 1978) have measured increases in the concentrations of extracellular and intracellular AMP concomitant with the development of the cyanide-insensitive respiration. Stimulation of the alternative oxidase by AMP was only observed with mitochondria of *Acanthamoeba castellanii* (Edwards & Lloyd, 1978b). Our results now imply that an AMP effect could be of more widespread occurrence, but might have been obscured by saturating intramitochondrial AMP concentrations. Clearly submitochondrial particles needed AMP, whereas mitochondria showed no response, unless these were prepared by a method that involved lower sucrose concentrations in the media for mitochondrial preparation and respiratory measurements. This also indicates that AMP may easily be lost from the mitochondria and is probably located on the outer side of the inner membrane.

Finally our data do not support or refute the belief that the development of an alternative oxidase is related to a modification of the inner mitochondrial membrane (Nakamura & Asahi, 1976; Waring & Laties, 1977a,b). If such a modification occurs in *M. tomentosa*, it is certainly without effect on the kinetics of the main respiratory pathway, as these remain unchanged whether the pathway operates alone or in combination with the alternative oxidase. The affinity of the alternative oxidase for succinate, however, seems to be altered when present together with the main chain or when present alone. A change in affinity is not observed with the mitochondria, and this 'anomaly' certainly needs further investigation.

We thank Dr. L. Hanssens for reading and discussing the manuscript. Part of this work was supported by a grant to J. K. from the Belgian Ministry of National Education.

References

- Bendall, D. S. & Bonner, W. D., Jr. (1971) *Plant Physiol.* **47**, 236–245
- Bonner, W. D., Jr. & Rich, P. R. (1978) *Proc. FEBS Meet. 11th* **49**, 149–158
- Chance, B. (1965) *J. Gen. Physiol.* **49**, 163
- De Pierre, J. W. & Ernster, L. (1977) *Annu. Rev. Biochem.* **46**, 201–262
- De Troostenbergh, J. C. (1978) Ph.D. Thesis, University of Louvain
- Edwards, S. W. & Lloyd, D. (1978a) *Proc. FEBS Meet. 11th* **49**, 31–37
- Edwards, S. W. & Lloyd, D. (1978b) *Biochem. J.* **174**, 203–211
- Hanssens, L. & Verachtert, H. (1976) *J. Bacteriol.* **125**, 829–836
- Hanssens, L., D'Hondt, E. & Verachtert, H. (1974) *Arch. Microbiol.* **98**, 339–349
- Hanssens, L., von Jagow, G. & Verachtert, H. (1978) *Proc. FEBS Meet. 11th* **49**, 47–53
- Henry, M. F. (1976) Ph.D. Thesis, University of Louvain
- Henry, M. F. & Nyns, E. J. (1975) *Sub-Cell. Biochem.* **4**, 1–65
- Henry, M. F., Bonner, W. D., Jr. & Nyns, E. J. (1977) *Biochim. Biophys. Acta* **460**, 94–100
- Keilin, D. & King, T. E. (1958) *Nature (London)* **181**, 1520–1522
- Lance, C., Dizengremel, P. & Chauveau, M. (1978) *Proc. FEBS Meet. 11th* **49**, 133–139
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lyr, H. & Schewe, T. (1975) *Acta Biol. Med. Germ.* **34**, 1631–1641
- Matsunaki, S. & Conti, S. F. (1966) *Plant Physiol.* **41**, 1370–1375
- Mitchell, P. (1975a) *FEBS Lett.* **56**, 1–6
- Mitchell, P. (1975b) *FEBS Lett.* **59**, 137–139
- Moore, A. L. & Rupp, H. (1978) *FEBS Lett.* **93**, 73–77
- Munn, E. A. (1974) *The Structure of Mitochondria*, pp. 316–319, Pergamon Press, Oxford
- Nakamura, K. & Asahi, T. (1976) *Arch. Biochem. Biophys.* **174**, 393–401
- Rich, P. R. & Bonner, W. D., Jr. (1977) *Plant Physiol. Suppl. Vol.* **56**, 59
- Rich, P. R. & Moore, A. L. (1976) *FEBS Lett.* **65**, 339–344
- Schonbaum, G. R., Bonner, W. D., Jr., Storey, B. T. & Bahr, J. T. (1971) *Plant Physiol.* **47**, 124–128
- Sharpless, T. K. & Butow, R. A. (1970) *J. Biol. Chem.* **245**, 58–70
- Sherald, J. L. & Sisler, H. D. (1970) *Plant Physiol.* **46**, 180–182
- Vanderleyden, J., Hanssens, L. & Verachtert, H. (1978) *J. Gen. Microbiol.* **105**, 63–68
- Van Der Plas, L. H. W. (1977) Ph.D. Thesis, University of Amsterdam
- Waring, A. J. & Laties, G. G. (1977a) *Plant Physiol.* **60**, 5–10
- Waring, A. J. & Laties, G. G. (1977b) *Plant Physiol.* **60**, 11–16
- Wedding, R. T., McReady, C. C. & Harley, J. L. (1973) *New Phytol.* **72**, 1–13
- Wilson, S. B. & Bonner, W. D., Jr. (1970) *Plant Physiol.* **46**, 31–35
- Yu, C. A., Yu, L. & King, E. (1977) *Biochem. Biophys. Res. Commun.* **78**, 259–265