Substrate Kinetics of the Alternative Oxidase of *Neurospora crassa*

Jos VANDERLEYDEN, Christine PEETERS and Hubert VERACHTERT

Laboratory for Industrial Microbiology and Biochemistry, University of Leuven, 3030 Heverlee-Louvain, Belgium

and Helmut BERTRAND

Department of Biology, University of Regina, Regina, Saskatchewan, Canada S4S 0A2

(Received 18 December 1979)

The kinetics of the succinate oxidation by cyanide-sensitive and cyanide-insensitive submitochondrial particles of *Neurospora crassa* cells suggest that both respiratory pathways use the same complex II. This is confirmed by comparing the kinetics of the reductase activities of the isolated succinate-ubiquinone oxidoreductase (complex II) of cyanide-sensitive and cyanide-insensitive cells respectively. No alternative-oxidase activity was found to be associated with the isolated complex II of cyanide-insensitive cells.

The mechanism of the alternative oxidase present in some organisms remains elusive (Lloyd & Edwards, 1978). Bendall & Bonner (1971) summarized the various hypotheses and concluded that the most likely candidate for the alternative oxidase itself is an iron-sulphur species. Rich & Bonner (1978) collected some evidence, mainly based on e.p.r. studies, against a succinate-reducible iron-sulphur centre as a possible candidate for the alternative oxidase. They suggested an autoxidizable quinone species, which interacts with the quinone pool associated with the succinate dehydrogenase, as another interesting possibility for the alternative oxidase. This is currently considered as the most likely candidate for the alternative oxidase (Moore & Rupp, 1978; Rich, 1978; Vanderleyden *et al.*, 1980a).

In the present paper, we report a kinetic study of succinate oxidation in submitochondrial particles of wild-type *Neurospora crassa* cells, grown in the presence of chloramphenicol. In wild-type *N. crassa*, the alternative oxidase is repressed under normal growth conditions (Edwards & Rosenberg, 1976), whereas growth in the presence of chloramphenicol induces the alternative oxidase (Edwards *et al.*, 1974). Attention is also focused on the enzymic properties of the isolated succinate-ubiquinone oxidoreductase (complex II) of cyanide-sensitive and cyanide-insensitive cells.

Methods and Materials

Preparations

Micro-organism and growth of cells. The strain of *Neurospora crassa* used throughout this study was

wild-type 74-OR23-1A (abbreviated 74A). Cultures were grown at 23°C in liquid Vogel's medium (Vogel, 1964) in 30-litre jars, aerated by water suction. Cells in the absence of chloramphenicol were grown for 24 h. Cells in the presence of 4 mg of chloramphenicol/ml were grown for 36 h.

Preparation of mitochondria and submitochondrial particles. Mycelium was disrupted with a grind mill (Weiss *et al.*, 1970), and mitochondria were prepared as previously described (Bertrand & Pittenger, 1969). Submitochondrial particles were obtained by sonication of the mitochondria (Werner, 1977).

Preparation of the succinate-ubiquinone oxidoreductase (complex II). Complex II of *N. crassa* was isolated as described by Ziegler & Rieske (1967) for ox heart mitochondria: 5 g wet wt. of a mitochondrial paste was used.

Analytical procedures

Determination of K_m values. The apparent K_m values for succinate in submitochondrial particles were estimated as described previously (Vanderleyden *et al.*, 1979). The reaction mixture (final volume 3 ml) consisted of 0.44 M-sucrose, 10 mM-Tris/HCl, 10 mM-MgSO₄, 10 mM-KH₂PO₄, 2 mM-EDTA and 0.2% (w/v) bovine serum albumin, pH 7.3. The K_m values for succinate of isolated complex II were estimated by varying the concentration of succinate in the reaction mixture (see below).

Determination of the reductase activities of complex II. The succinate-ubiquinone reductase activities of complex II were measured by following the reduction of 2,6-dichlorophenol-indophenol by

dihydrourubiquinone-2 (Hatefi & Stiggall, 1978). The succinate dehydrogenase activities of complex II were measured by following the phenazine methosulphate-mediated reduction of dichlorophenol-indophenol (Hatefi & Stiggall, 1978). The reductase activities were measured with a Beckman Acta V spectrophotometer.

Materials. Ubiquinone-2 was generously given by Dr. J. Sippel, St. Louis University School of Medicine, and duroquinone was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results and Discussion

The apparent K_m values for succinate of the cytochrome oxidase-mediated and the alternative-oxidase-mediated succinate oxidase of *N. crassa*, grown in the presence of chloramphenicol, are given in Table 1. The estimations were done with submitochondrial particles. 5'-GMP was added to the reaction mixture, since we demonstrated that the alternative oxidase requires nucleoside 5'-monophosphates to exhibit activity in submitochondrial particles (Vanderleyden *et al.*, 1980b).

Apparently, there is a difference in the kinetics of succinate oxidation through the alternative oxidase and the cytochrome oxidase (first line of Table 1). 5'-GMP has no effect on the apparent K_m value for succinate of the cytochrome oxidase-mediated succinate oxidase. When the apparent K_m values, however, are measured in the presence of ferri-cytochrome *c* (lower line of Table 1), they are found to be identical for both oxidases. This is due to the increase in the K_m value for succinate of the cytochrome oxidase-mediated succinate oxidase in the presence of cytochrome *c*. These data indicate that, in the submitochondrial particles, cytochrome *c* is the rate-limiting step for the oxidation of succinate through the cytochrome oxidase, while the alternative oxidase is still in excess for the oxidation of succinate through the alternative pathway. Since

the K_m values for succinate under appropriate conditions are identical for both pathways, it can be concluded that the K_m value obtained for succinate is determined either by a common succinate dehydrogenase or by another compound common to both pathways. This compound should be part of complex II, because the branchpoint between the two pathways is at the ubiquinone-cytochromes *b* level (Rich & Bonner, 1978).

In *Moniliella tomentosa* (Vanderleyden *et al.*, 1979), the K_m value for succinate of the alternative oxidase was under all circumstances (mitochondria, submitochondrial particles) higher than the K_m value for succinate via the normal respiratory pathway. In order to explain this difference between the two organisms we have to isolate complex II of *M. tomentosa* as we did for *N. crassa* (see below). Nevertheless, it should be noted that the procedures used for the isolation of submitochondrial particles were quite different for the two organisms. Furthermore, we have to consider that the alternative oxidase of *N. crassa* is fully repressed under normal growth conditions (Edwards & Rosenberg, 1976), whereas the alternative oxidase of *M. tomentosa* can account for almost 50% of the total respiratory activity under normal growth conditions in the stationary growth phase (Hanssens & Verachtert, 1976).

Rich (1978) reported the isolation of a partially purified alternative oxidase of *Arum maculatum* mitochondria by using deoxycholate. The isolated fraction, however, showed activity only towards quinols as electron donors. We have shown, however, that use of deoxycholate (Vanderleyden *et al.*, 1980b) results in the absolute requirement for nucleoside 5'-monophosphates in *N. crassa*. Moreover, with *M. tomentosa* (Vanderleyden *et al.*, 1980a), we have shown that quinol oxidation may be the result of autoxidation mechanisms. Hence an alternative approach was now used to attempt to isolate the alternative oxidase as a functional unit. The activity of the alternative oxidase was followed through the different steps necessary to isolate complex II of mitochondria of *N. crassa* grown in the presence of chloramphenicol. Succinate was used as electron donor. In the isolation procedure used (Ziegler & Rieske, 1967) it appeared that succinate oxidation through the alternative pathway was lost in the first step [(NH₄)₂SO₄/cholate fractionation]. Recombination of the supernatant with the pellet failed to restore the activity. Additions of ubiquinone-2, duroquinone and/or 5'-GMP to the reaction mixture were also unsuccessful. This is due to the inactivation of the alternative oxidase itself, since the succinate-ubiquinone reductase (complex II) was found to be active throughout the complete fractionation procedure (see below).

Finally, we compared the enzymic properties of

Table 1. K_m values for succinate of the succinate oxidase in submitochondrial particles of *Neurospora crassa*, grown in the presence of chloramphenicol

The K_m values for succinate were determined from the rate of O₂ consumption after small additions of succinate to the reaction mixture (see the Methods and Materials section). The alternative oxidase was measured in the presence of 1 mM-KCN, and the normal oxidase in the presence of 2.6 mM-salicylhydroxamic acid, besides the additions indicated.

Additions	K_m (mM-succinate)	
	Alternative oxidase	Normal oxidase
5'-GMP	0.74	0.22
—	—	0.21
Ferrocycytochrome <i>c</i>	0.75	0.68

Table 2. K_m values for succinate of the succinate-ubiquinone reductase (complex II) of *Neurospora crassa*, grown in the absence and the presence of chloramphenicol

The K_m values of succinate were determined from the rate of dichlorophenol-indophenol reduction after small additions of succinate to the reaction mixture (see the Methods and Materials section). The reductions were measured in 1 ml sample cells with a Beckman Acta V spectrophotometer.

Assay	Chloramphenicol	...	K_m (mM-succinate)	
			Absent	Present
Ubiquinone-2-dichlorophenol-indophenol			1.4	1.5
Phenazine methosulphate-dichlorophenol-indophenol			2.1	2.1

the isolated complex II of *N. crassa* cells, grown in the absence and presence of chloramphenicol. The K_m values of succinate for the isolated complex II of the two types of cells were estimated in two different ways, as summarized in Table 2. The isolated preparations showed no reductase activity unless ubiquinone or phenazine methosulphate was added to the reaction mixture. This means that the isolated complexes were completely depleted of the naturally occurring quinone(s) during the isolation procedure. The K_m values obtained for complex II of cyanide-sensitive and cyanide-insensitive cells are almost identical. This means that there is no difference in enzymic properties between the two types of cells, with respect to this complex II. The succinate-ubiquinone reductase activities of both preparations were completely sensitive to 1 mM-thenoyltrifluoroacetone, a specific inhibitor of the reductase activity of complex II (Hatefi & Stiggall, 1978). When duroquinone was substituted for ubiquinone-2 in the succinate-quinone-dichlorophenol-indophenol assay, none of the preparations showed reductase activity. The isolated complex II of cyanide-insensitive cells was tested subsequently with respect to the effect of nucleoside 5'-monophosphates and

salicylhydroxamic acid on the succinate-ubiquinone reductase activity. Nucleoside 5'-monophosphates were shown to stimulate the alternative-oxidase activity of *N. crassa* (Vanderleyden *et al.*, 1980b), whereas salicylhydroxamic acid is known to be an inhibitor of the alternative oxidase (Schonbaum *et al.*, 1971). None of these showed any effect on the succinate-ubiquinone reductase activities. This would indicate that salicylhydroxamic acid and the nucleoside 5'-monophosphates act on the O_2 side of ubiquinone in the alternative pathway.

References

- Bendall, D. S. & Bonner, W. D., Jr. (1971) *Plant Physiol.* **47**, 236-245
- Bertrand, H. & Pittenger, T. H. (1969) *Genetics* **61**, 643-659
- Edwards, D. L. & Rosenberg, E. (1976) *Eur. J. Biochem.* **62**, 217-221
- Edwards, D. L., Rosenberg, E. & Maroney, P. (1974) *J. Biol. Chem.* **249**, 3551-3556
- Hanssens, L. & Verachtert, H. (1976) *J. Bacteriol.* **125**, 829-836
- Hatefi, Y. & Stiggall, D. L. (1978) *Methods Enzymol.* **53D**, 21-27
- Lloyd, D. & Edwards, S. W. (1978) *Proc. FEBS Meet.* **11th** **49**, 1-10
- Moore, A. L. & Rupp, H. (1978) *FEBS Lett.* **93**, 73-77
- Rich, P. R. (1978) *FEBS Lett.* **96**, 252-256
- Rich, P. R. & Bonner, W. D., Jr. (1978) *Proc. FEBS Meet.* **11th** **49**, 149-158
- Schonbaum, G. R., Bonner, W. D., Jr., Storey, B. T. & Bahr, J. T. (1971) *Plant Physiol.* **47**, 124-128
- Vanderleyden, J., Kurth, J. & Verachtert, H. (1979) *Biochem. J.* **182**, 437-443
- Vanderleyden, J., Van Den Eynde, E. & Verachtert, H. (1980a) *Biochem. J.* **186**, 309-316
- Vanderleyden, J., Peeters, C., Verachtert, H. & Bertrand, H. (1980b) *Biochem. J.* in the press
- Vogel, H. J. (1964) *Am. Nat.* **98**, 435-446
- Weiss, H., von Jagow, G., Klingenberg, M. & Bucher, Th. (1970) *Eur. J. Biochem.* **14**, 75-82
- Werner, S. (1977) *Eur. J. Biochem.* **79**, 103-110
- Ziegler, D. & Rieske, J. S. (1967) *Methods Enzymol.* **10**, 231-235