Stimulation of the Alternative Oxidase of *Neurospora crassa* by Nucleoside Phosphates

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The alternative-oxidase-mediated succinate oxidase activity of *Neurospora crassa* decreases drastically when mitochondria are fractionated into submitochondrial particles or treated with deoxycholate. The activity, however, can be completely restored in the presence of nucleoside 5'-monophosphates. The purine nucleoside 5'-monophosphates are more effective than the pyrimidine homologues. 5'-GMP gives a 10-fold stimulation of the alternative-oxidase-mediated succinate oxidase activity in submitochondrial particles. A comparison is made with the results obtained earlier with *Moniliella tomentosa* [Hanssens & Verachtert (1976) *J. Bacteriol.* **125**, 825–835; Vanderleyden, Van Den Eynde & Verachtert (1980) *Biochem. J.* **186**, 309–316].

The nature of the alternative oxidase, which is present in mitochondria of various plants and eukaryotic micro-organisms (Henry & Nyns, 1975; Solomos, 1977), has remained elusive. It has been suggested that the mechanism of the alternative oxidase in all organisms is very similar, in spite of some individual differences (Vanderleyden *et al.*, 1980).

The alternative oxidase of *Neurospora crassa* was first detected in cytochrome-deficient mutants (Tissières *et al.*, 1953) and subsequently found to be inducible in wild-type strains (Lambowitz & Slayman, 1971; Edwards & Rosenberg, 1976). Several mutants deficient in alternative-oxidase activity have been isolated (Edwards *et al.*, 1976; Szakacs, 1978). A limitation in the biochemical study of the alternative oxidase of *N. crassa* is the loss of its activity after the fractionation of the mitochondria into submitochondrial particles (Bohrer, 1975) or after treatment of the mitochondria with detergents, in order to purify the alternative oxidase.

In previous reports (Hanssens & Verachtert, 1976; Vanderleyden *et al.*, 1979), we have described a stimulation of the alternative oxidase of *Moniliella tomentosa* by purine nucleoside 5'-phosphates in mitochondria and submitochondrial particles. In mitochondria, however, the stimulation can be masked, depending on the isolation procedure for the mitochondria (Vanderleyden *et al.*, 1980). Therefore three methods were described to reveal the

effect of the nucleoside 5'-phosphates on the alternative oxidase (Vanderleyden *et al.*, 1980).

In the present paper we report that the mitochondrial alternative oxidase of *N. crassa* can also be stimulated by nucleoside 5'-monophosphates, especially by 5'-GMP. Moreover, they are required to restore the alternative-oxidase activity in detergent-treated mitochondria and submitochondrial particles, which explains why its activity was assumed to be lost after fractionation of mitochondria into particles.

Experimental

Preparations

Strains and culture conditions. Three strains of *Neurospora crassa* were used: wild-type 74-OR23-1A (abbreviated 74A); a cytoplasmic cytochrome-deficient mutant *mi-3* (Mitchell *et al.*, 1953); a nuclear cytochrome-deficient mutant *cya-4-23*, described by Bertrand *et al.* (1977). Strains 74A and *mi-3* were obtained from the Fungal Genetics Stock Center (FGSC; Humboldt State University Foundation, Arcata, CA, U.S.A.). All the cultures were grown at 25°C in liquid Vogel's medium (Vogel, 1964) as described previously (Bertrand *et al.*, 1968; Bertrand & Pittenger, 1969). The mutant strains were grown for 48 h. The wild-type strain was grown for 12 h, followed by a 6 h period in the presence of 4 mg of chloramphenicol/ml [74A(CAP) in Table 1].

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

Analytical procedures

O₂ consumption. O₂ consumption was measured polarographically with a Clark-type oxygen electrode. The reaction mixture 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.

Determination of K_m values. The apparent K_m for 5'-GMP was determined as described previously (Vanderleyden *et al.*, 1979).

Protein determination. Mitochondrial- and submitochondrial-particle protein was determined by the biuret method (Layne, 1957), with bovine serum albumin as standard.

Materials. The nucleoside 5'-monophosphates were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results

Comparison of the succinate oxidase activities of mitochondria and submitochondrial particles of Neurospora crassa

Table 1 shows the succinate oxidase activities of various *N. crassa* strains, possessing both the

normal and the alternative respiratory chain. Table 1 (upper part) shows that 1 mM-5'-AMP stimulates moderately the alternative oxidase in mitochondria, in contrast with submitochondrial particles. In submitochondrial particles its activity in the absence of 5'-AMP is very low and appears to be lost during the fractionation of the mitochondria into particles, but it is markedly stimulated when 5'-AMP is added to the reaction mixture.

Furthermore, Table 1 (lower part) shows that 5'-AMP has no stimulatory effect on the oxidase activity of the normal cytochrome chain (measured in the presence of 2.6 mM-salicylhydroxamic acid). A minor inhibitory effect can be observed, at least in mitochondria. In addition, it is clear that exogenous ferricytochrome *c* has no effect on the alternative-oxidase-mediated succinate oxidase activity.

Effect of detergent solubilization on the alternative-oxidase-mediated succinate oxidase activity of Neurospora crassa mitochondria

Rich (1978) reported that the rates of the cyanide-insensitive oxidation of both succinate and NADH in *Arum maculatum* mitochondria decreased rapidly when the deoxycholate concentration in the reaction medium was increased. In contrast, we demonstrated with *Moniliella tomentosa* mitochondria that the alternative-oxidase-mediated succinate oxidase activity measured in the presence of 1 mM-KCN and 1 mM-5'-AMP can be enhanced by up to 400% of the original activity by the addition of deoxycholate in a certain detergent/protein ratio (Vanderleyden *et al.*, 1980).

Table 1. Succinate oxidase activities of mitochondria and submitochondrial particles of *Neurospora crassa*

The oxidase activities were measured polarographically with 10 mM-succinate as substrate. The reaction mixture (final volume 3 ml, temperature 25°C, pH 7.3) consisted of 0.44 M-sucrose, 10 mM-KH₂PO₄, 10 mM-MgSO₄, 2 mM-EDTA, 0.2% (w/v) bovine serum albumin and mitochondria (Mito, 0.6–0.8 mg of protein) or submitochondrial particles (ETP, 0.7–0.9 mg of protein). The same data were obtained whether ferricytochrome *c* was added before or after 5'-AMP. Abbreviation used: CAP, chloramphenicol. Additions were made sequentially to the same reaction mixture in the order of listing from top to bottom of the column.

Strain ...	Oxidase activities (ng-atoms of O/min per mg of protein)					
	74A (CAP)		mi-3		cya-4-23	
	Mito	ETP	Mito	ETP	Mito	ETP
Succinate	376	80	138	204	328	192
KCN (1 mM)	61	17	21	11	36	8
5'-AMP (1 mM)	78	63	39	30	61	21
Ferricytochrome <i>c</i> (0.2 mg)	78	63	39	20	61	21
Salicylhydroxamic acid (2.6 mM)	10	6	0	0	0	0
Succinate	385	102	126	225	366	177
Salicylhydroxamic acid (2.6 mM)	139	85	116	213	260	165
5'-AMP (1 mM)	118	85	116	213	248	165
Ferricytochrome <i>c</i> (0.2 mg)	134	137	146	285	248	186
KCN (1 mM)	16	0	0	0	0	0

Fig. 1 illustrates the effect of increasing deoxycholate concentrations on the alternative-oxidase-mediated and the cytochrome oxidase-mediated succinate oxidase activity of *N. crassa* mitochondria. As the detergent concentration is increased, the rate of the cyanide-insensitive oxidation of succinate, measured in the presence of 1 mM-KCN, decreases rapidly. However, when 1 mM-5'-AMP is added to the reaction mixture, the rate of the cyanide-insensitive oxidation of succinate is completely restored and even enhanced by up to 60% of the original activity. The increased activity is completely sensitive to 2.6 mM-salicylhydroxamic acid. At a detergent/protein ratio above 0.9:1 (w/w), both the alternative-oxidase-mediated and cytochrome oxidase-mediated succinate oxidase activities decrease rapidly. Preliminary results have shown that the succinate-cytochrome *c* reductase of these mitochondria is still active at a detergent/protein ratio of 4:1 (w/w).

Fig. 1. Effects of deoxycholate on the alternative oxidase and the normal respiratory pathway of mitochondria of *Neurospora crassa* (74A), grown in the presence of chloramphenicol

The oxidase activities were measured as indicated in the legend to Table 1. The appropriate amount of detergent was added, followed by 1 mM-KCN (alternative oxidase) or 2.6 mM-salicylhydroxamic acid (normal respiratory pathway), 10 mM-succinate and 1 mM-5'-AMP. The original specific activities of the alternative-oxidase-mediated and the cytochrome oxidase-mediated succinate oxidases were 53 and 155 ng-atoms of O/min per mg of protein respectively. O, Alternative oxidase; ●, alternative oxidase in the presence of 1 mM-5'-AMP; □, normal respiratory chain in the presence or absence of 1 mM-5'-AMP.

Specificity of nucleoside 5'-monophosphates to stimulate the alternative oxidase of Neurospora crassa

Testing the specificity of four different nucleoside 5'-monophosphates with respect to their effect on the alternative-oxidase-mediated succinate oxidase activity reveals that the alternative oxidase has the highest activity in the presence of 5'-GMP. 5'-AMP, 5'-UMP and 5'-CMP are much less effective than 5'-GMP. With these nucleoside 5'-monophosphates, the increased activity of the alternative-oxidase-mediated succinate oxidase activity is completely inhibited by 2.6 mM-salicylhydroxamic acid. The same results were obtained with NADH as the reducing substrate (results not shown).

The stimulatory effect of these nucleoside 5'-monophosphates on the alternative oxidase is more pronounced in detergent-treated mitochondria (Table 2, column b) and submitochondrial particles (Table 2, column c) than in intact mitochondria (Table 2, column a) of wild-type *N. crassa* cells, grown in the presence of chloramphenicol. The data in Table 2 reveal also that the alternative-oxidase activities of the mutant strains (Table 2, columns d and e) are much less stimulated by the nucleoside 5'-monophosphates (especially 5'-GMP) than is the inducible alternative-oxidase activity (Table 2, column c). This is observed with submitochondrial particles. The apparent *K_m* value for 5'-GMP of the alternative oxidase, estimated with submitochondrial particles of wild-type *N. crassa* cells grown in

Table 2. Specificity of nucleoside 5'-monophosphates with respect to their stimulatory effect on the alternative-oxidase-mediated succinate oxidase activity of *Neurospora crassa* mitochondria and submitochondrial particles

The oxidase activities were measured as indicated in the legend of Table 1. (a) Mitochondria of strain 74A, grown in the presence of chloramphenicol; (b) as (a), in the presence of deoxycholate (0.75 mg/mg of protein); (c) submitochondrial particles of strain 74A, grown in the presence of chloramphenicol; (d) submitochondrial particles of strain *mi-3*; (e) submitochondrial particles of strain *cya-4-23*. The original activities (ng-atoms of O/min per mg of protein) of the different assays were: (a), 52; (b), 8; (c), 16; (d), 13; (e), 11. The concentration of all nucleoside 5'-monophosphates added was 1 mM.

Additions	Stimulation (%)				
	(a)	(b)	(c)	(d)	(e)
None	—	—	—	—	—
5'-GMP	520	1000	1020	420	380
5'-AMP	180	340	400	280	260
5'-UMP	160	240	410	160	180
5'-CMP	—	170	240	140	140

the presence of chloramphenicol, was found to be $31\text{ }\mu\text{M}$ -5'-GMP.

Discussion

This is the first time that the stimulatory effect of nucleoside 5'-monophosphates on the alternative-oxidase activity of *N. crassa* has been reported. These observations explain why previous workers (Bohrer, 1975) have stated that the alternative-oxidase activity of wild-type *N. crassa*, grown in the presence of chloramphenicol, is lost during the fractionation of mitochondria into particles (see also Table 1). It is possible now to start some preparative work on the alternative oxidase of *N. crassa*, in order to isolate and to purify the oxidase. The stimulation of the alternative oxidase activity of *N. crassa* by nucleoside 5'-monophosphates, by analogy with *Moniliella tomentosa* (Hanssens & Verachtert, 1976; Vanderleyden *et al.*, 1980), substantiates experimentally that the mechanism of the alternative oxidase of both micro-organisms is very similar, if not identical.

It is not clear why the alternative-oxidase activity in the submitochondrial particles of the mutant strains is less stimulated by the nucleoside 5'-monophosphates than is the inducible alternative-oxidase activity (Table 2). It can only be assumed that the component on which the nucleoside 5'-monophosphates acts is more labile in the mutant strains during the preparation of submitochondrial particles.

The specificity of the nucleoside 5'-monophosphates to stimulate the alternative-oxidase activity of *N. crassa* is not restricted to the purine nucleoside phosphates, as observed with the alternative oxidase of *M. tomentosa* (Vanderleyden *et al.*, 1980). Nonetheless, 5'-GMP was found to be much more effective. The apparent K_m value of 5'-GMP for the alternative oxidase of *N. crassa* is almost identical with the apparent K_m value of 5'-AMP for the alternative oxidase of *M. tomentosa* ($25\text{ }\mu\text{M}$ -5'-AMP) (Vanderleyden *et al.*, 1979). The mechanism by which the nucleoside 5'-monophosphates stimulate the alternative oxidase is not clear. Charge-transfer

interactions with quinones have been suggested (Vanderleyden *et al.*, 1980).

With respect to the composition of the buffer used for the isolation of mitochondria, it should be noted that *M. tomentosa* mitochondria lose all the alternative-oxidase activity in 0.44 M -sucrose, which was the concentration used for the isolation of *N. crassa* mitochondria, unless exogenous 5'-AMP was added.

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