Nature of the Effect of Adenosine 5'-Monophosphate on the Cyanide-Insensitive Respiration in Mitochondria of *Moniliella tomentosa*

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The alternative oxidase of *Moniliella tomentosa* mitochondria is stimulated by 5'-AMP. This effect may be masked, depending on the isolation procedure of the mitochondria. The preparation of submitochondrial particles results in the expression of the 5'-AMP effect. Two more methods are now described to reveal the 5'-AMP effect whenever it would be masked: (1) switching on the myokinase activity of the mitochondria to deplete them of endogenous 5'-AMP; (2) using detergents (sodium dodecyl sulphate, sodium deoxycholate) in a controlled detergent:protein ratio, or chloroform. The alternative oxidase of detergent-solubilized mitochondria was somewhat less selective towards nucleotides than were intact mitochondria. The effect of nucleotides on quinol oxidation by mitochondrial preparations and on quinol autoxidation was also studied. Mitochondrial oxidation of succinate by the alternative oxidase and autoxidation of quinols behaved similarly in the presence of certain nucleotides. Both reactions were stimulated. Both reactions were also inhibited by salicylhydroxamic acid. These effects on quinol oxidation disappeared when bovine serum albumin or mitochondrial proteins were present. From the results obtained it is not possible to exclude quinol autoxidation as a final step of the alternative oxidase.

The key problem of the alternative oxidase that is present in a variety of higher plants and eukaryotic micro-organisms (Lloyd, 1974; Henry & Nyns, 1975; Solomos, 1977) is still the nature of the component(s) involved in this pathway. It can be speculated, however, that the mechanism of the alternative oxidase in all organisms is very similar, in spite of some peculiarities. One of these peculiar aspects, found in mitochondria of *Moniliella tomentosa* (Hanssens & Verachtert, 1976), *Euglena gracilis* (Sharpless & Butow, 1970) and *Acanthamoeba castellanii* (Edwards & Lloyd, 1978), is the stimulation of the alternative oxidase by purine nucleoside 5'-phosphates.

We have demonstrated that the stimulation of the alternative oxidase in *M. tomentosa* mitochondria by 5'-AMP can be masked, depending on the isolation procedure for these mitochondria (Vanderleyden *et al.*, 1979). Consequently it was suggested that the 5'-AMP effect could be a more widespread feature of the alternative oxidase, even in these organisms where it has never been demonstrated.

Disruption of mitochondria (Vanderleyden *et al.*, 1979) is a good method to check whether a 5'-AMP effect would be masked. The present paper describes two additional methods to reveal an eventual 5'-AMP effect on the alternative oxidase.

Since it is agreed that quinones are the most likely candidates for the alternative-oxidase O₂-consuming component (Rich, 1978; Rich & Bonner, 1978a,b; Moore & Rupp, 1978), the oxidation of quinols by intact and partially detergent-solubilized mitochondria of *M. tomentosa* is reported here. In addition, the effects of different nucleotides on the alternative-oxidase-mediated oxidation of succinate and quinol are reported. Emphasis is focused on the effects of nucleotides and salicylhydroxamic acid on the autoxidation of quinols.

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 as described by Vanderleyden *et al.* (1979). The standard medium was supplemented with 4 mg of chloramphenicol/ml to obtain cyanide-insensitive cells.

Preparation of mitochondria. Mitochondria were prepared from 24 h-grown cells as described by Vanderleyden *et al.* (1979).

Preparation of reduced quinol substrates. Menadiol was prepared from the corresponding quinone by using sodium dithionite, as described by Rich (1978). Duroquinol was prepared from the corresponding duroquinone by using ZnCl_2 , as described by Bohrer (1975).

Analytical procedures

O_2 consumption. O_2 consumption was measured polarographically as described by Vanderleyden *et al.* (1979). O_2 uptake by mitochondria with NADH or succinate as substrate was measured in sucrose buffer [0.8 M-sucrose, 5 mM-Tris/HCl, 5 mM-KCl, 20 mM- KH_2PO_4 , 0.3% (w/v) bovine serum albumin, pH 6.8], with the use of 0.65–0.9 mg of mitochondrial protein. O_2 uptake by mitochondria in the presence of quinols, and the autoxidation of quinols, were measured in a medium containing 50 mM-4-morpholine-ethanesulphonic acid at pH 6.0, as described by Rich (1978).

Determination of K_m values. The K_m values for succinate, O_2 and 5'-AMP were determined as described by Vanderleyden *et al.* (1979).

Enzyme assays. D-Amino acid oxidase (from pig kidney, EC 1.4.3.3) was assayed from its rate of O_2 consumption in the presence of D-alanine, as described by Boulanger & Osteux (1965). L-Amino acid oxidase (from *Crotalus adamanteus*, EC 1.4.3.2) was assayed from its rate of O_2 consumption in the presence of L-leucine, as described by Gale (1965). D-Glucose oxidase (from *Aspergillus niger*, EC 1.1.3.4) was assayed from its rate of O_2 consumption in the presence of D-glucose, as described by Bergmeyer & Bernt (1965).

Protein determination. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard (Sigma Chemical Co., grade V). Mitochondrial proteins were solubilized with 0.2% (w/v) deoxycholate before determination.

Materials. Menadione, 2'-, 3'- and 5'-AMP, ADP, ATP, 5'-GMP, 5'-CMP, 5'-UMP, D-amino acid oxidase, L-amino acid oxidase and D-glucose oxidase were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Duroquinone was obtained from K & K Chemicals, Plainview, NY, U.S.A. Salicyl-hydroxamic acid was obtained from Aldrich-Europe Division, Janssen Pharmaceutica, Belgium. All chemicals used were of analytical grade.

Results

Detection and depletion of intramitochondrial 5'-AMP with mitochondrial myokinase

Hanssens & Verachtert (1976) demonstrated that the alternative oxidase of *M. tomentosa* mitochondria exhibited only a very low activity unless exogenous purine nucleoside 5'-monophosphates and to some extent 5'-diphosphates were added to the reaction mixture. However, when mitochondria were isolated by a modified procedure (Vanderleyden *et al.*, 1979), the alternative oxidase operated always at full capacity whether or not exogenous 5'-AMP was present in the reaction mixture. It was assumed that intramitochondrial 5'-AMP was lost in the procedure of Hanssens & Verachtert (1976). Vanderleyden *et al.* (1979) also showed that mitochondrial 5'-AMP was lost during the preparation of submitochondrial particles. To confirm these results, Fig. 1 shows that endogenous 5'-AMP can also be removed by switching on the mitochondrial myokinase activity.

Fig. 1(a) shows that exogenous 5'-AMP has no effect on the alternative oxidase activity of *M. tomentosa* mitochondria isolated by the modified procedure. When these mitochondria are preincubated with ATP or ADP, in the presence of succinate or NADH (results not shown) and before the addition of cyanide to the reaction mixture, the alternative oxidase needs exogenous 5'-AMP (Figs. 1b, 1c) or 5'-GMP (results not shown) to operate. Incubation with ATP or ADP, in the presence of succinate or NADH (results not shown), but after the addition of cyanide, had only a minor effect on the requirement of the alternative oxidase for exogenous 5'-AMP or 5'-GMP (Figs. 1d, 1e). This experiment suggests that, when mitochondria respire in the presence of exogenous ATP, the mitochondrial 5'-AMP pool is converted into ADP via the myokinase activity of the mitochondria.

When ADP is present, this is phosphorylated to ATP via oxidative phosphorylation, associated with the main cytochrome chain, which is present in these mitochondria, as shown by the oxidation rates in the absence or presence of cyanide (Fig. 1a). ATP again will react with the 5'-AMP present, via the myokinase activity. ATP alone has no stimulatory effect on the alternative oxidase (Hanssens & Verachtert, 1976). When ATP is added in the presence of cyanide, the mitochondrial 5'-AMP will be converted only into ADP, owing to the inhibition of the respiratory chain, resulting in an accumulation of ADP. Hanssens & Verachtert (1976) demonstrated that ADP stimulates the alternative oxidase, although to a lesser extent than 5'-AMP. The accumulation of ADP, however, is sufficient to explain the lack of further stimulation by added 5'-AMP. As has been observed with submitochondrial particles

Fig. 1. Depletion of intramitochondrial 5'-AMP by the addition of ATP or ADP

The succinate oxidase activity was measured polarographically. The mitochondria were isolated by the modified procedure (Vanderleyden *et al.*, 1979). 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, 20 mM-KH₂PO₄, 0.3% (w/v) bovine serum albumin. At the points indicated, additions were made of 0.65 mg of mitochondrial protein (Mito), 20 mM-succinate (Suc), 1 mM-5'-AMP, 0.3 mM-ATP, 0.12 mM-ADP, 1 mM-KCN and 2.6 mM-salicylhydroxamic acid (SHAM). The values above the traces are the rates of O₂ consumption in ng-atoms of O/min per mg of protein.

Table 1. Effect of nucleotides on the alternative-oxidase-mediated succinate oxidase activity of *M. tomentosa* mitochondria, depleted of endogenous 5'-AMP

The oxidase activities were measured polarographically as indicated in Fig. 1. The mitochondria were depleted of 5'-AMP either through the activity of the mitochondrial myokinase with the addition of ATP (a) as indicated in Fig. 1, or through the addition of deoxycholate to the mitochondrial suspension in a detergent: protein ratio of 3:1 (b); 1 mM-KCN was added to ensure that all electron flow was proceeding via the alternative oxidase. The initial concentration of succinate was 20 mM; the concentration of nucleotides was 1 mM.

Additions	Succinate oxidase activity (ng-atoms of O/min per mg of protein)		Stimulation (%)	
	(a)	(b)	(a)	(b)
	(a)	(b)	(a)	(b)
None	30	25	—	—
5'-AMP	135	188	450	705
5'-AMP	110	210	370	840
2'-AMP	30	70	None	280
3'-AMP	30	58	None	230
ADP	110	115	370	460
ATP	30	37	None	150
5'-CMP	32	25	None	None
5'-UMP	34	25	None	None
Salicylhydroxamic acid	0	0	—	—
5'-AMP + salicylhydroxamic acid	0	0	—	—

(Vanderleyden *et al.*, 1979), it is noteworthy that by removing the mitochondrial 5'-AMP pool via the myokinase activity *in vivo*, the stimulation of the

alternative oxidase is again restricted to purine nucleoside 5'-monophosphates and 5'-diphosphates and cannot be observed with the 2'- or 3'-phosphate

esters of the purine nucleosides nor with any of the pyrimidine nucleotides, as illustrated in Table 1(a). This is in agreement with the results obtained by Hanssens & Verachtert (1976) with mitochondria lacking the 5'-AMP pool. The corresponding nucleosides and nitrogen bases were not tested because of their poor solubility in water.

Detection and depletion of intramitochondrial 5'-AMP through detergent treatment

Rich (1978) demonstrated that a selective solubilization of the alternative oxidase of *Arum maculatum* mitochondria could be obtained by adding deoxycholate to a mitochondrial preparation in a carefully controlled detergent:protein ratio. The alternative oxidase was suggested to be a quinol oxidase, since the alternative oxidase of mitochondria in the presence of deoxycholate showed only activity towards quinols as substrates.

Fig. 2 illustrates the effects of detergent solubilization on the alternative-oxidase-mediated oxidation of

succinate and NADH by *M. tomentosa* mitochondria. Both NADH oxidase and succinate oxidase activities decrease rapidly with increasing detergent concentration. Sodium deoxycholate, sodium dodecyl sulphate and chloroform were tested. In the presence of sodium dodecyl sulphate and chloroform, only the succinate oxidase activity was measured. The decrease in NADH oxidase and succinate oxidase activity in the presence of deoxycholate has been reported by Rich (1978) in *Arum maculatum* mitochondria. With respect to the succinate oxidase activity, however, we show here that the activity can be restored and even enhanced, with 1 mM-5'-AMP, up to 400, 240 and 150% of the original activity, depending on the detergent used. The restored activity is completely inhibited by salicylhydroxamic acid, an indication that the increased oxygen uptake is caused by the alternative oxidase. Restoration of succinate oxidase activity with 5'-AMP, however, is limited to a certain detergent:protein ratio. A further increase in the detergent concen-

Fig. 2. Effects of (a) sodium deoxycholate, (b) sodium dodecyl sulphate and (c) chloroform on the NADH oxidase and succinate oxidase activities of *M. tomentosa* mitochondria

The oxidase activities were measured polarographically as indicated in the legend to Fig. 1. The appropriate amount of detergent was added, followed by 1 mM-KCN or 2.6 mM-salicylhydroxamic acid to ensure that all electron flow was proceeding via the alternative respiratory chain or the normal cytochrome chain. After incubation for 1 min at 25°C, substrate was added. The original specific activities of the alternative oxidase activities were: NADH oxidase, 472 ng-atoms of O/min per mg of protein; succinate oxidase, 92 ng-atoms of O/min per mg of protein. The original specific activity of the cytochrome-mediated succinate oxidase activity was 52 ng-atoms of O/min per mg of protein. Initial concentrations of substrates were 1.0 mM-NADH or 20 mM-succinate. □, NADH oxidase activity in the presence of 1 mM-KCN; ■, NADH oxidase activity in the presence of 1 mM-KCN and 1 mM-5'-AMP; ○, succinate oxidase activity in the presence of 1 mM-KCN; ●, succinate oxidase activity in the presence of 1 mM-KCN and 1 mM-5'-AMP; △, succinate oxidase activity in the presence of 2.6 mM-salicylhydroxamic acid.

tration probably dissociated or dissolved the components involved in the cyanide-insensitive electron transfer from succinate to O_2 so that they no longer interact. Nevertheless the data shown in Fig. 2 clearly illustrate that addition of deoxycholate, sodium dodecyl sulphate or chloroform to a mitochondrial suspension, in a controlled detergent:protein ratio, depleted mitochondria, among other things, of 5'-AMP, without any damage to the alternative oxidase itself. The different behaviour of the NADH oxidase and succinate oxidase activities towards deoxycholate most likely can be explained by the different localization of the two dehydrogenases with respect to the inner mitochondrial membrane. De Pierre & Ernster (1977) showed that the succinate dehydrogenase is localized at the inner-facing side of the inner mitochondrial membrane. The NADH dehydrogenase of yeast mitochondria for the oxidation of external added NADH was shown to be localized at the outer-facing side of the inner mitochondrial membrane (von Jagow & Klingenberg, 1970). The increase in the alternative succinate oxidase activity in the presence of exogenous 5'-AMP with increasing detergent concentration is more difficult to explain. The apparent K_m for 5'-AMP at the optimal deoxycholate:protein ratio was found to be $6\mu M$. With intact mitochondria and submitochondrial particles an apparent K_m value of $25\mu M$ -5'-AMP was estimated (Vanderleyden *et al.*, 1979). This would indicate a higher accessibility of 5'-AMP to the alternative oxidase in the presence of detergents. The apparent K_m value for succinate at the optimal deoxycholate:protein ratio was found to be 0.9 mM . With intact mitochondria and submitochondrial particles, apparent K_m values of 8.0 mM and 2.4 mM -succinate respectively were estimated (Vanderleyden *et al.*, 1979). The apparent K_m value for O_2 was found to be almost the same in intact mitochondria, detergent-solubilized mitochondria and submitochondrial particles ($11\mu M$ - O_2).

The effects of other nucleotides on the restoration of the alternative-oxidase activity in detergent-solubilized mitochondria were then investigated.

Effects of nucleotides on the alternative-oxidase-mediated succinate oxidation of partially detergent-solubilized mitochondria of M. tomentosa

The specificity of 5'-AMP, 5'-GMP and to some extent ADP and GDP in restoring the alternative-oxidase-mediated succinate oxidation of 5'-AMP-depleted mitochondria through detergent treatment (Table 1b) is almost the same as was found with the 5'-AMP-depleted mitochondria by using the mitochondrial myokinase (Table 1a), disruption into submitochondrial particles (Vanderleyden *et al.*, 1979) or alternative isolation techniques for the mitochondria (Hanssens & Verachttert, 1976). Slight differences, however, are the minor stimulations by

ATP, 2'-AMP and 3'-AMP with the detergent-treated mitochondria. Furthermore, it is confirmed that salicylhydroxamic acid completely blocks the O_2 uptake whether or not nucleotides are added to the reaction mixture. It might be concluded from these results that partial solubilization of the mitochondria with deoxycholate extended the specificity of nucleotides, to stimulate the alternative oxidase, to the complete set of purine nucleotides. 2'-GMP, 3'-GMP and GTP were not tested, but since 5'-GMP and 5'-AMP stimulated the alternative oxidase in an identical manner, they are believed to behave identically with the adenine derivatives.

It is noteworthy that the alternative oxidase of *M. tomentosa* mitochondria may be inhibited by sodium deoxycholate, sodium dodecyl sulphate or chloroform without disturbing the normal respiratory chain, at least at a certain detergent:protein ratio. This is shown in Fig. 2. The inhibition of the alternative oxidase may be reasonably explained by the loss of 5'-AMP.

Effect of nucleotides on the alternative-oxidase-mediated quinol oxidation by M. tomentosa mitochondria and on the autooxidation of quinols

Since quinols are found as electron donors capable of providing electrons at or close to the alternative-oxidase O_2 -consuming step (Rich & Bonner, 1978a,b; Moore & Rupp, 1978; Huq & Palmer, 1978; Hanssens *et al.*, 1978), we decided to investigate the effect of nucleotides on the oxidation of quinols in cyanide-insensitive mitochondria of *M. tomentosa*. Table 2 illustrates the O_2 -consumption rates of cyanide-insensitive mitochondria in the presence of menadiol, under the different conditions tested. It is clear that mitochondria enhance menadiol oxidation. It is doubtful, however, that the observed quinol oxidation in the presence of mitochondria is due to a specific quinol oxidase, since replacement of mitochondrial protein by bovine serum albumin in the reaction mixture also resulted in an increased O_2 uptake. Both were directly proportional to the concentration of protein used. Therefore we believe that the O_2 consumption in the presence of mitochondrial protein is due to an increased autooxidation of menadiol. Moreover, the oxidation of menadiol in the presence of intact and detergent-solubilized mitochondria was not affected by the addition of 5'-AMP to the reaction mixture, and 2.6 mM -salicylhydroxamic acid inhibited only moderately. The same results were obtained with the oxidation of menadiol in the presence of the $100\,000g$ supernatant, obtained after centrifugation of a deoxycholate-solubilized mitochondrial preparation, at a detergent:protein ratio of 4:1 (w/w). This method was described by Rich (1978) to purify the alternative oxidase of *Arum maculatum* mitochondria.

Table 2. *Effect of 5'-AMP on the alternative-oxidase-mediated oxidation of menadiol by M. tomentosa mitochondria*
 O_2 uptake was measured polarographically in a medium containing 50 mM-4-morpholine-ethanesulphonic acid, pH 6.0 (final volume 3 ml, temperature 25°C). The initial concentration of menadiol was 0.5 mM; the concentration of 5'-AMP added was 1 mM. Salicylhydroxamic acid (SHAM) was added at a concentration of 2.6 mM, and deoxycholate (DOC) at 1.25 mg. Except where otherwise stated, mitochondria (Mito) were added at 0.50 mg of protein.

Additions	O_2 consumption (ng-atoms of O/min per mg of protein)	Stimulation (%)
None	67	—
Mito (0.25 mg of protien)	180	270
Mito (0.50 mg of protein)	323	500
Mito + 5'-AMP	323	500
Bovine serum albumin (2 mg)	239	350
Albumin (3 mg)	294	440
Albumin (3 mg) + 5'-AMP	294	440
Albumin (3 mg) + SHAM	158	230
Albumin (3 mg) + 5'-AMP + SHAM	175	260
Mito + DOC	285	440
Mito + DOC + 5'-AMP	285	440
Mito + SHAM	211	300
Mito + DOC + SHAM	210	300
Mito + DOC + 5'-AMP + SHAM	205	300

Table 3. *Effect of various nucleotides on the autoxidation of menadiol and duroquinol*

Autoxidation was measured polarographically in a medium containing 50 mM-4-morpholine-ethanesulphonic acid, pH 6.0 (final volume 3 ml, temperature 25°C). The initial concentrations of menadiol and duroquinol were respectively 0.5 mM and 1 mM. The concentration of nucleotides added was 1 mM; salicylhydroxamic acid was added in a final concentration of 2.6 mM.

Additions	Rate of O_2 consumption (ng-atoms of O/min)		Stimulation (%)	
	Menadiol	Duroquinol	Menadiol	Duroquinol
None	49	7.5	—	—
5'-AMP	110	15	225	200
5'-GMP	250	24	500	300
2'-AMP	80	Not measured	165	—
3'-AMP	82	Not measured	165	—
ADP	220	Not measured	450	—
ATP	245	Not measured	500	—
5'-CMP	50	8	None	None
5'-UMP	52	8	None	None
5'-AMP + salicylhydroxamic acid	8	0	—	—
5'-GMP + salicylhydroxamic acid	8	0	—	—
Salicylhydroxamic acid	0	0	—	—

In this context, autoxidation of quinols was studied. It was very unexpected to discover a stimulation of the autoxidation of menadiol by different nucleotides in the absence of proteins. Moreover, the autoxidation of menadiol in the absence of proteins was inhibited by salicylhydroxamic acid. This is shown in Table 3. The same results were obtained with the autoxidation of duroquinol. It is remarkable that only purine nucleotides increased the autoxidation of quinols. This corresponds to the effects of nucleotides on the alternative-oxidase-mediated succinate oxidation by detergent-treated mitochondria. One difference, however, is the fact that ATP and ADP stimulated the autoxidation more than 5'-AMP.

Effect of 5'-AMP on the activity of FAD-requiring enzymes

Since flavoproteins may be involved in the oxidation-reduction reactions through semiquinol species, we investigated the effects of nucleotides and salicylhydroxamic acid on some FAD enzymes such as D-amino acid oxidase, L-amino acid oxidase and glucose oxidase. The first two enzymes are active through semiquinol intermediates and the last functions solely between the fully oxidized and fully reduced forms of FAD (Palmer & Massey, 1968). It was found that the activity of the enzymes was not increased in the presence of 1 mM-5'-AMP; 2.6 mM-salicylhydroxamic acid inhibited the amino acid oxi-

dases by up to 40%, but glucose oxidase was not affected. These results might be a preliminary indication that nucleotides are not acting at the level of flavoproteins.

Discussion

The results obtained in this work demonstrate that the stimulation of the cyanide-insensitive respiration of *M. tomentosa* mitochondria by nucleotides may be masked by the existence of an intramitochondrial pool of nucleotides, especially 5'-AMP or 5'-GMP. It would be interesting to investigate whether such a masking effect exists in other organisms showing cyanide-insensitive respiration. Three methods to reveal the 5'-AMP effect may be helpful: (1) the preparation of submitochondrial particles (Vanderleyden *et al.*, 1979); (2) the use of detergents such as sodium deoxycholate and sodium dodecyl sulphate, or chloroform; (3) the activation of the mitochondrial myokinase. In *M. tomentosa* mitochondria it is now clear that the alternative oxidase can be stimulated by 5'-AMP, 5'-GMP, ADP and GDP. Unless Mg^{2+} is present, the last two are less active. In the presence of Mg^{2+} , ATP and GTP are also active (Hanssens & Verachtert, 1976). This seems to indicate that purine nucleosides esterified with phosphate at C-5' are specific requirements. With detergent-treated mitochondria, however, purine nucleosides esterified with phosphate at C-2' or -3' also showed stimulation of the alternative oxidase. The components of the alternative oxidase are located in the mitochondrial inner membrane (Vanderleyden *et al.*, 1979). It is possible that, after treatment of the mitochondria with deoxycholate, the arrangement or conformation of proteins might be altered to such an extent that nucleotides with an ester bond other than at C-5' will also interact.

A very interesting observation from this study is the inhibition of the autoxidation of menadiol and duroquinol by salicylhydroxamic acid and its stimulation by purine nucleotides. The stimulation of quinol autoxidation by proteins should also be emphasized. In this connection the stimulation of quinol oxidation by mitochondria or mitochondrial extracts is not a definite proof that enzymic reactions are occurring. It would have been interesting to know whether the solubilized quinol oxidase obtained by Rich (1978) was still inhibited by salicylhydroxamic acid.

The inhibition of quinol autoxidation by salicylhydroxamic acid could be explained by the polyfunctional hydrogen-binding capacity of hydroxamic acids (Schonbaum, 1973; Rich *et al.*, 1978). When mitochondrial proteins or bovine serum albumin are added, however, the oxidation of menadiol is only moderately inhibited by salicylhydroxamic acid. One explanation might be that proteins

interfere with the complex formed between hydroxamic acid and quinols. If the alternative oxidase appears to be a quinol oxidase, it is not clear why the alternative-oxidase-mediated oxidation of NADH or succinate is inhibited by salicylhydroxamic acid, whereas salicylhydroxamic acid does not inhibit the oxidation of menadiol in the presence of mitochondria, partially detergent-solubilized mitochondria or mitochondrial extracts. The stimulation of quinol autoxidation by purine nucleotides is more difficult to explain. Possibly charge-transfer interactions are involved (Slifkin, 1971). Such an hypothesis can be tested experimentally, since a charge-transfer absorption band should be detected. The differences observed between the oxidation of menadiol in the presence or absence of proteins towards the effect of purine nucleotides might also be explained by the interference of proteins in the complex-formation between purine nucleotides and quinols.

The effects of salicylhydroxamic acid and nucleotides are very similar on the autoxidation of quinols and on the oxidation of succinate (or NADH) via the alternative oxidase of *M. tomentosa*. The effects on quinol oxidation, however, disappear when mitochondria or bovine serum albumin are present. As bovine serum albumin enhanced the autoxidation, it is believed that menadiol oxidation in the presence of mitochondria can also be explained as stimulation of its autoxidation. If the alternative oxidase appears to be an autoxidizable pool of quinols (Rich & Bonner, 1978a) linked to the dehydrogenases of the substrates, it should then be assumed that salicylhydroxamic acid and purine nucleotides interfere at a level before its autoxidation. An indication in favour of the latter possibility is the fact that 5'-AMP has the highest stimulating effect on the alternative oxidase and ATP the lowest, whereas on autoxidation the reverse was found.

Another possibility to explain the enhanced oxidation of menadiol by mitochondria could be the presence of a true quinol oxidase, which is not inhibited by salicylhydroxamic acid nor stimulated by purine nucleotides.

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