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AMP-dependence of the cyanide-insensitive pathway in the respiratory chain of *Paramecium tetraurelia*

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1. The AMP-dependent stimulation of the cyanide-insensitive respiration of *Paramecium* mitochondria was investigated. 2. The nucleotides exhibiting a stimulatory effect on the cyanide-insensitive oxidation of pyruvate (+malate) in a medium supplemented with EDTA or carboxyatractyloside were, in decreasing order of efficiency, AMP, GMP, IMP, UMP and TMP. On the other hand, ADP, ATP and cyclic AMP were ineffective. In the presence of carboxyatractyloside, addition of AMP to *Paramecium* mitochondria incubated with pyruvate (+malate) led to an increase in membrane potential. 3. In the absence of light, the photoactivable derivative of AMP, 3'-{4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl}-AMP (NAP_4-AMP) added to *Paramecium* mitochondria opposed the stimulatory effect of AMP on the cyanide-insensitive respiration; the K_i for NAP_4-AMP was much lower than the K_m for AMP, 0.2 μM compared with 120 μM. The ADP-stimulated respiration was not affected. 4. Photoirradiation of *Paramecium* mitochondria in the presence of NAP_4-AMP resulted in irreversible inhibition of the AMP-stimulated cyanide-insensitive respiration. No effect on the ADP-stimulated respiration was observed. 5. A heat-labile cyanide-insensitive ubiquinol oxidase was extracted from *Paramecium* mitochondria with the detergent *NN*-dimethyl-*N*-(3-laurylamidopropyl)amine oxide. The quinol oxidase activity was slightly stimulated by AMP.

The mitochondria of *Paramecium tetraurelia* possess, besides the common cytochrome aa_3 (cytochrome *c* oxidase) that belongs to the main respiratory pathway, two alternative pathways to O_2 (Doussière *et al.*, 1979*a*,*b*). The three pathways differ in their sensitivity to cyanide. Cytochrome aa_3 is inhibited by low concentrations of cyanide ($K_i \simeq 10\,\mu$M). One of the two alternative oxidases is insensitive to millimolar concentrations of cyanide; it is inhibited by low concentrations of salicylhydroxamic acid (K_i 120 μM), and activated by AMP (K_m 120 μM); it is probably branched at ubiquinone (coenzyme Q) and fed by reducing equivalents coming from NAD-linked substrates and succinate. The second alternative oxidase behaves as a cytochrome *b* oxidase; it is less sensitive to cyanide (K_i 50 μM) than is cytochrome aa_3, and less sensitive-salicylhydroxamic acid (K_i 700 μM) than the first alternative oxidase. Recently, doubts have been expressed concerning the enzymic nature of the process involved in the cyanide-insensitive transfer of an electron from ubiquinone to O_2 (Rustin *et al.*, 1983). These doubts were based on the report (Vanderleyden *et al.*, 1980*b*) that autoxidation of quinols, such as menadiol and duroquinol, is inhibited by salicylhydroxamic acid and activated by AMP; the activating effect of AMP was explained by a charge transfer between the purine ring of AMP and the quinone ring of ubiquinone.

In the present paper, we show that AMP and to a lesser extent GMP and IMP activate the cyanide-insensitive respiration of *Paramecium* mitochondria by binding to a specific component of the respiratory chain.

Abbreviations used: LAPAO, *NN*-dimethyl-*N*-(3-laurylamidopropyl)amine oxide; NAP_4-AMP, 3'-{4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl}-AMP; TPP^+, tetramethylphosphonium.

Materials and methods

Materials

4-Amino[2,3-^{3}H]butyric acid was obtained from the Commissariat à l'Energie Atomique (Saclay,

France). All other chemicals were of reagent-grade quality. Ubiquinone-1 was a gift from Dr. Brubalker and Dr. Leuenberger of Hoffmann-La Roche (Basel, Switzerland). Ubiquinone-1 indicates the quinone with a side chain containing one prenyl unit in accordance with the recommended ubiquinone-*n* nomenclature (Crane & Barr, 1971).

Biological preparations

The wild-type strain of *Paramecium tetraurelia* used in the present experiments originated from stock d4-2 (termed in accordance with the nomenclature of Sonneborn, 1970). The conditions of growth and the preparation of mitochondria were as previously described (Doussière *et al.*, 1979*a*). The mitochondria were finally suspended in a medium containing 0.5M-mannitol, 0.1% bovine serum albumin, 5mM-Mops (4-morpholinepropanesulphonate) and 1mM-EDTA, final pH7.3. In specific experiments bearing on oxidation of ubiquinone-1, the mutant Cl_1, deprived of cytochrome aa_3, was used; it is a double mutant carrying a nuclear mutation Cl_1 and a mitochondrial mutation M^{cl} (Doussière *et al.*, 1979*a*).

Photolabelling assays

NAP_4-AMP (Fig. 1) was synthesized by the same method as that described for synthesis of NAP_4-ADP, starting from 4-aminobutyric acid (Lauquin *et al.*, 1978); the 3'-secondary alcohol function of the ribose moiety of AMP was linked to the carboxy group of the butyryl derivative of the 4-fluoro-3-nitrophenyl azide in the presence of carbonyl di-imidazole in dry dimethylformamide. In photoinactivation experiments, *Paramecium* mitochondria in the presence of NAP_4-AMP were subjected to repeated flashes of light (Ultrablitz-Matador III G.m.b.H). In photolabelling assays, [^{3}H]NAP_4-AMP was used.

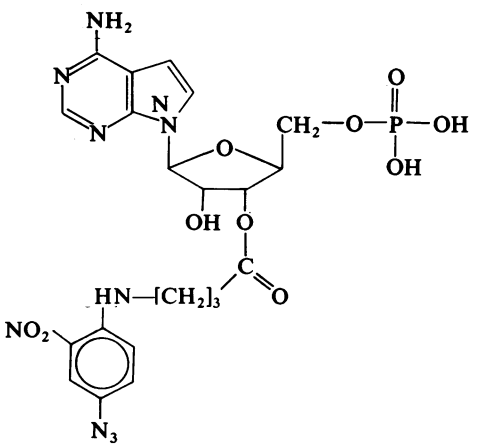

Fig. 1. *Structure of NAP_4-AMP*

Measurement of respiration

Respiration of mitochondria was measured polarographically with a Clark electrode at 22°C. The medium contained 0.5M-mannitol, 0.05% bovine serum albumin, 5mM-$MgCl_2$ or 1mM-EDTA and 10mM-potassium phosphate buffer, pH7.3, final volume 1.5ml. KCN was freshly prepared for each experiment, and its pH was adjusted to 7.4–7.6.

Oxidation of ubiquinol-1

Ubiquinol-1 was prepared by dithionite reduction of ubiquinone-1, followed by diethyl ether extraction as described by Rich (1978). The rate of oxidation of ubiquinol-1 was measured spectrophotometrically as described by Crane & Barr (1971) by using a $\Delta\varepsilon$ (oxidized − reduced) value of $12500\,M^{-1}\cdot cm^{-1}$ at 275nm.

Extraction of membrane proteins from Paramecium mitochondria by LAPAO

Paramecium mitochondria were diluted to a concentration of 15mg of protein/ml in the same medium as that used for isolation. The mitochondrial suspension was supplemented with LAPAO to a final concentration of 0.1% (w/v). After 5min at 0°C, the suspension was centrifuged at 100000*g* for 15min; a clear supernatant, called S1, was recovered, and the pellet was treated once again with 1.5% LAPAO for 5min at 0°C. Another centrifugation under the same conditions yielded a second pellet and a supernatant S2. Both supernatants were examined for their oxidase activity with respect to ubiquinol-1.

Determination of membrane potential $\Delta\psi$

$\Delta\psi$ was determined by a method based on the measurement of the amount of accumulated TPP^+, a permeant cation, by respiring mitochondria. The TPP^+ concentration in the incubation medium was measured with a TPP^+ electrode prepared as described by Kamo *et al.* (1979). $\Delta\psi$ was derived from the equation:

$$\Delta\psi = 2.3\frac{RT}{F}\cdot\log\left(\frac{v}{V}\right) - 2.3\frac{RT}{F}\cdot\log\left(10^{F\Delta E/2.3RT} - 1\right)$$

where v is the volume of the matrix space of mitochondria, V the external volume, and ΔE the difference in the electrode potential (Muratsugu *et al.*, 1977). The uptake of TPP^+ was corrected for TPP^+ binding in the presence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The volume of the matrix space was derived from the difference between the volumes occupied by ^3H_2O (total space) and by [^{14}C]-

sucrose (external space plus intermembrane space); the average value found in three assays, 0·7 μl/mg of protein, was used for calculation.

Results

Conditions for a specific stimulatory effect of AMP on the cyanide-insensitive respiration of Paramecium mitochondria

Paramecium mitochondria have a small content of adenine nucleotide (less than 2 nmol/mg of protein) compared with mammalian mitochondria [e.g. 15 nmol/mg of protein for rat liver mitochondria (Duée & Vignais, 1969)]. Previous experiments performed with an $MgCl_2$-supplemented medium have shown that AMP added to *Paramecium* mitochondria stimulates the oxidation of NAD-dependent substrates and succinate with a K_m of 120 μM (Doussière *et al.*, 1979*b*).

As shown in Fig. 2(*a*), in the presence of carboxyatractyloside, an inhibitor of ADP/ATP transport, AMP still enhanced the cyanide-resistant respiration. Under these conditions, ADP had no effect. The stimulatory effect of ADP on the cyanide-insensitive respiration of the fungus *Moniliella tomentosa* (Vanderleyden *et al.*, 1980*a*) may be explained by oxidative phosphorylation of the added ADP at the level of the first phosphorylation site, before the branching of the cyanide-insensitive chain. Stimulation of the cyanide-resistant respiration of *Paramecium* mitochondria by AMP was also observed when $MgCl_2$ was replaced by 1 mM-EDTA, a condition that prevents accumulation of ADP by transphosphorylation. The stimulatory effect of AMP in an EDTA-supplemented medium makes the autoxidation of mitochondrial quinols by cations contributed by AMP highly improbable; besides, EDTA prevents the adenylate kinase-mediated transphosphorylation of AMP into ADP by traces of ATP that could have been released from mitochondria.

Nucleotide specificity

Besides AMP, the purine nucleotides GMP and IMP were able to activate the cyanide-insensitive respiration, although to a lesser extent than AMP (Fig. 2*b*); the pyrimidine nucleotides CMP and UMP had a slight effect, indicating that the purine

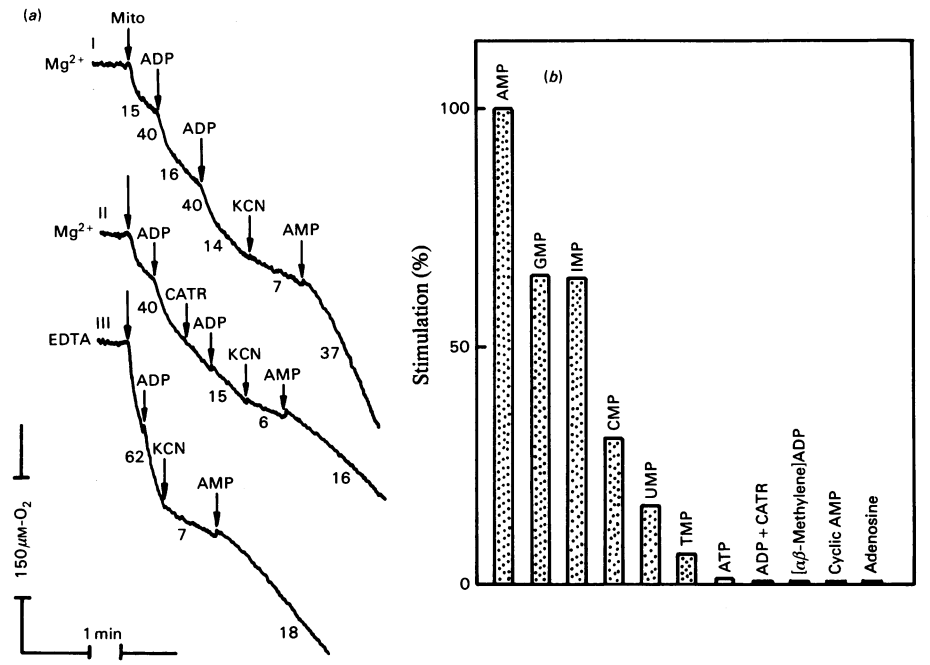

Fig. 2. *Effects of AMP and other nucleotides on the cyanide-insensitive respiration of Paramecium mitochondria*
(*a*) Effects of $MgCl_2$ and EDTA on the AMP-dependent stimulation of the cyanide-insensitive respiration. Mitochondria of *P. tetraurelia* (wild-type) (1 mg of protein) were incubated in the medium described in the Materials and methods section in the presence of 10 mM-pyruvate, 1 mM-malate and 1 mM-KCN. Each addition of ADP corresponded to a final concentration of 20 μM; AMP was added at the final concentration of 200 μM. In trace III $MgCl_2$ in the incubation medium was replaced by 1 mM-EDTA. The numbers on the traces are the rates of O_2 uptake in nmol/min per mg of protein. Abbreviations: Mito, Mitochondria; CATR, carboxyatractyloside. (*b*) Compared stimulatory effects of AMP and other nucleotides. The nucleotides were added at the final concentration of 1 mM. Because AMP was the most efficient nucleotide, the stimulatory effect of AMP was chosen as reference (100% stimulation).

ring is not an absolute requirement for a nucleotide to stimulate the cyanide-insensitive respiration. TMP, ATP, adenosine, cyclic AMP and the phosphonic analogue of ADP [αβ-methylene]ADP were ineffective.

Difference in the response of the membrane potential to addition of AMP and ADP

The segment of the respiratory chain NADH-ubiquinone, on which the cyanide-insensitive oxidase is branched, is electrogenic, whereas the branched oxidase itself is non-electrogenic (Doussière *et al.*, 1979*b*). The responses of the membrane potential in *Paramecium* mitochondria supplemented with pyruvate (+ malate) and then with cyanide to the addition of ADP and AMP in the presence or in the absence of carboxyatractyloside are illustrated in Fig. 3. As expected, on addition of ADP, there was a decrease in membrane potential, due to the energy-consuming reaction of

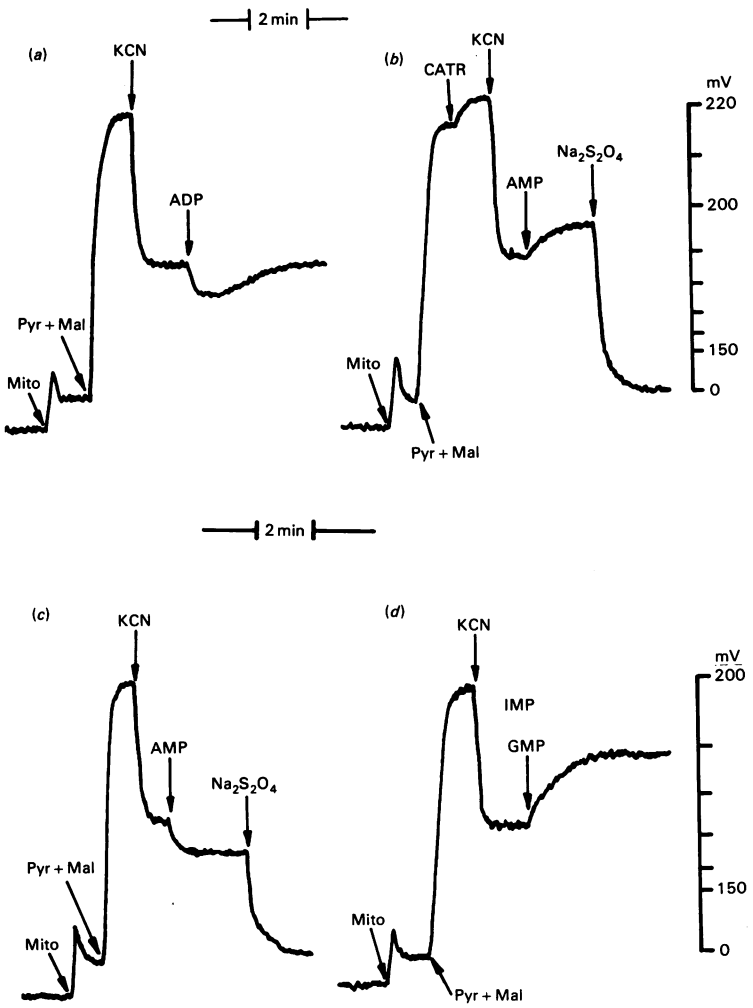

Fig. 3. *Effects of AMP and other nucleotides on membrane potential of Paramecium mitochondria incubated in the presence of KCN*

The medium used contained 0.5M-mannitol, 0.1% bovine serum albumin, 10mM-potassium phosphate, 5mM-$MgSO_4$, 10μM-TPP^+ Cl^- and 10mM-KCl, final pH 7.3. The amount of mitochondria was between 1.5 and 2mg of protein. Respiration was induced by addition of 10mM-pyruvate and 1mM-malate. The following final concentrations were used: carboxyatractyloside, 1μM; KCN, 1mM; AMP, 1mM; GMP, 0.5mM; IMP, 0.5mM; ADP, 0.5mM. The membrane potential corresponded to 95% of the protonmotive force generated by respiration, as measured by a subsequent addition of nigericin; this results from the presence of P_i in the medium and the proton-linked movement of P_i through the phosphate carrier. Abbreviations: Mito, mitochondria; Pyr, pyruvate; Mal, malate; CATR, carboxyatractyloside.

phosphorylation of ADP into ATP (Fig. 3*a*). In the presence of carboxyatractyloside addition of AMP resulted in an increase of membrane potential (Fig. 3*b*). The control assay carried out with AMP in the absence of carboxyatractyloside led to a decrease in membrane potential, which is similar to that observed after addition of ADP, and which is explained by the formation of ADP catalysed by adenylate kinase from the added AMP and traces of ATP released from mitochondria (Fig. 3*c*). On the other hand, replacement of AMP by IMP or GMP in the absence of carboxyatractyloside led to an increase in membrane potential, similar to that obtained by addition of AMP in the presence of carboxyatractyloside (Fig. 3*d*). In the presence of carboxyatractyloside, the newly generated ADP cannot enter the matrix space. The increase in membrane potential caused by addition of AMP in the presence of carboxyatractyloside is probably due to the acceleration of respiration in the NADH–ubiquinone segment of the respiratory chain, and the non-utilization of this increased potential for phosphorylation. This is corroborated by the assays carried out with IMP and GMP. This also suggests that AMP binds to a site located on the outer face of the mitochondrial inner membrane; in fact, if AMP had to penetrate the inner membrane to bind to its target site, it would be probably transphosphorylated into ADP by the transphosphorylation systems located within the matrix space, and this would lead to a decrease in membrane potential.

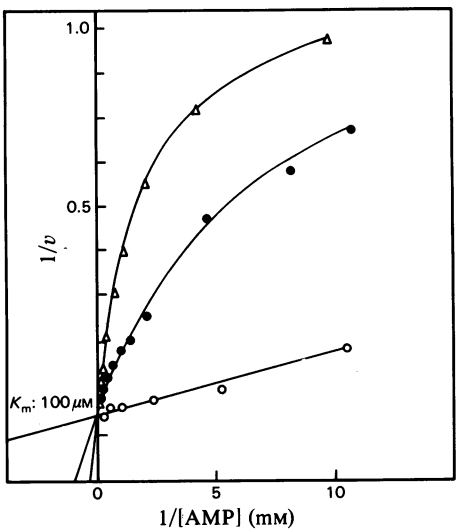

Fig. 4. *Effect of NAP_4-AMP on the rate of the AMP-stimulated respiration of Paramecium mitochondria in the absence of photoirradiation*

Paramecium mitochondria (0.8 mg of protein) were incubated in 1.5 ml of the respiration medium in the presence of 10 mM-pyruvate and 1 mM-malate and various concentrations of AMP. The concentrations of NAP_4-AMP used were: ○, none (control); ●, 2.2 μM; △, 5 μM. The reaction was carried out in a dim light; the incubation vessel was protected from light with aluminium foil. *v* is the rate of respiration expressed in nmol/min per mg of protein.

Effect of NAP_4-AMP, a photoactivable derivative of AMP, on the cyanide-insensitive respiration

In the absence of photoirradiation, NAP_4-AMP did not stimulate the cyanide-insensitive respiration as AMP did. On the contrary, NAP_4-AMP opposed the stimulatory effect of AMP; it had no effect on the ADP-stimulated respiration. The inhibition caused by NAP_4-AMP was relieved by increasing the concentration of AMP. The double-reciprocal plot of the rate of respiration for two fixed concentrations of NAP_4-AMP and increasing concentrations of AMP (Fig. 4) indicated that NAP_4-AMP behaved as a competitive inhibitor. The curvilinearity of the plots at low concentrations of AMP is typical of a high-affinity competitive inhibitor that binds more firmly than the substrate (Henderson, 1972); in fact, the K_i for NAP_4-AMP, 0.2 μM, was 600-fold lower than the K_m for AMP, 120 μM. Complementary assays (not shown) indicated that the NAP_4 moiety {4-[*N*-(4-azido-2-nitrophenyl)amino]butyric acid} had no effect on the AMP-stimulated respiration.

The effect of photoirradiation on the inhibition of respiration of *Paramecium* mitochondria by NAP_4-AMP is shown in Fig. 5(*a*) (trace II compared with trace I). ADP was first added in a limited concentration (70 μM) to induce State 3 respiration. After return to State 4, NAP_4-AMP was added at the final concentration of 5 μM, which is saturating (see above). The suspension in the cuvette was then photoirradiated by 20 flashes of visible light at intervals of 5 s. Photoirradiation with NAP_4-AMP did not modify the stimulatory effect of ADP on respiration, but inhibited by about 50% the stimulatory effect of AMP in the presence of cyanide. In contrast with the respiratory inhibition by NAP_4-AMP in the dark, which was relieved by increasing concentrations of AMP, inhibition by NAP_4-AMP after photoirradiation remained stable whatever the concentration of AMP used, which is in accordance with an all-or-none effect of photoirradiation on a fraction of the cyanide-insensitive respiratory chain.

As expected, on photoirradiation, a number of proteins were photolabelled, as revealed after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The radioactivity pattern showed three labelled peaks corresponding to M_r values of 50000, 35000 and 30000 (Fig. 5*b*). Preincubation with AMP before photoirradiation led to a

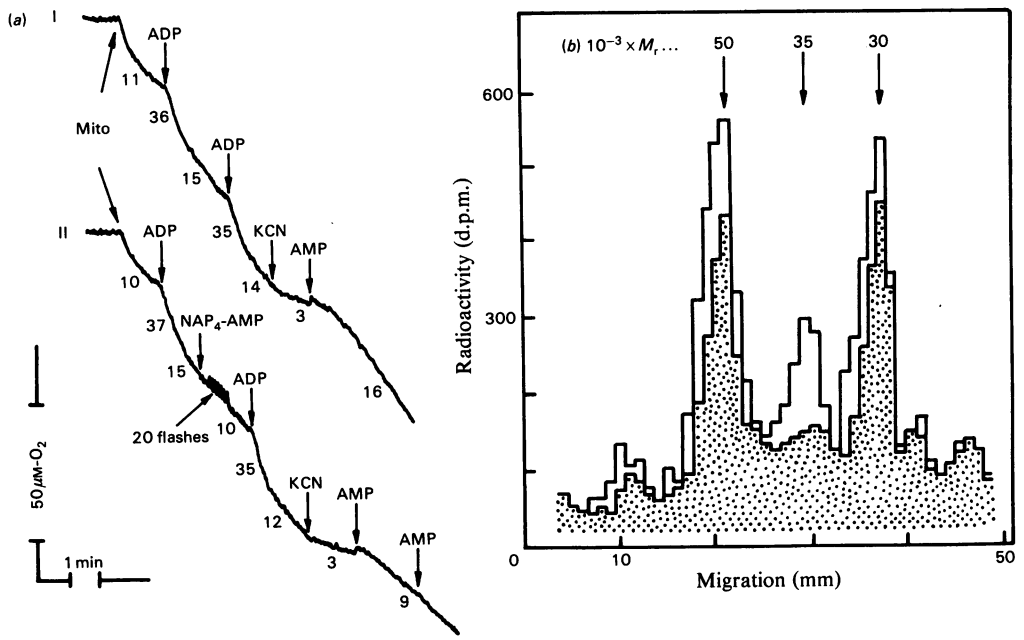

Fig. 5. *(a) Inactivation of the AMP-stimulated respiration of Paramecium mitochondria by photoirradiation with NAP_4-AMP and concomitant photolabelling and (b) gel-electrophoretic analysis of proteins from Paramecium mitochondria after photolabelling with [^3H]NAP_4-AMP*

(a) The medium was identical with that used in the experiment of Fig. 4. The assay was conducted with 1.2mg of mitochondrial protein. NAP_4-AMP was added at a final concentration of 5μM, followed by 70μM-ADP, 1mM-KCN, 1mM-AMP and finally 3mM-AMP. Abbreviation: Mito, mitochondria. *(b)* Mitochondria were incubated with 15μM-NAP_4-AMP with or without AMP (1mM). After photoirradiation, the mitochondria were sedimented by centrifugation and dissolved in 100μl of 2% sodium dodecyl sulphate and 1% 2-mercaptoethanol for gel electrophoresis. The Figure shows the profile of bound radioactivity: □, without AMP (control); ▨, with AMP.

decrease of the radioactivity in the two peaks corresponding to M_r 50000 and 35000. Because of lack of specific inhibitors, it was not possible to characterize further the specific AMP-binding component of the cyanide-resistant pathway. Salicylhydroxamic acid, an inhibitor of the cyanide-resistant respiration, preincubated with mitochondria decreased uniformly the radioactivity in the three peaks. This result may be explained by the fact that salicylhydroxamic acid behaves as a radical trap with respect to the nitrene radical formed from NAP_4-AMP during the course of photoirradiation; it therefore decreases the concentration in reactive nitrene.

Effect of AMP on oxidation of ubiquinol

Quinols are autoxidizable, especially at alkaline pH; autoxidation of menadiol and duroquinol have been reported to be enhanced by AMP (Vanderleyden *et al.*, 1980*b*). At pH 7.2 ubiquinol-1 is slowly oxidized in a mannitol medium; however, AMP had no stimulatory effect, and salicylhydroxamic acid had no inhibitory effect on the spontaneous oxidation of ubiquinol-1 (Fig. 6*a*). On the other hand, the O_2 uptake by oxidation of ubiquinol-1 was markedly accelerated of addition of mitochondria from the wild-type strain of *Paramecium* and moderately on addition of the mitochondria from the mutant Cl_1. In both cases, AMP markedly stimulated respiration, and this stimulatory effect was inhibited by salicylhydroxamic acid (Figs 6*b* and 6*c*).

The putative quinol oxidase activity responsible for the oxidation of ubiquinol-1 was extracted from the wild type-strain *Paramecium* mitochondria by the non-ionic detergent LAPAO. As described in the Materials and methods section, extraction was performed in two steps. The first step involved 0.1% detergent. Centrifugation yielded the supernatant S1 and a pellet, which was re-extracted with 1.5% detergent. A second centrifugation yielded the supernatant S2. An AMP-stimulated and cyanide-insensitive quinol oxidase activity was found in supernatant S2. This activity was slightly stimulated by AMP; it was proportional to the amount of mitochondrial extract used and was lost by thermal denaturation (Fig. 7). The effect of salicylhydroxamic acid could not be tested, be-

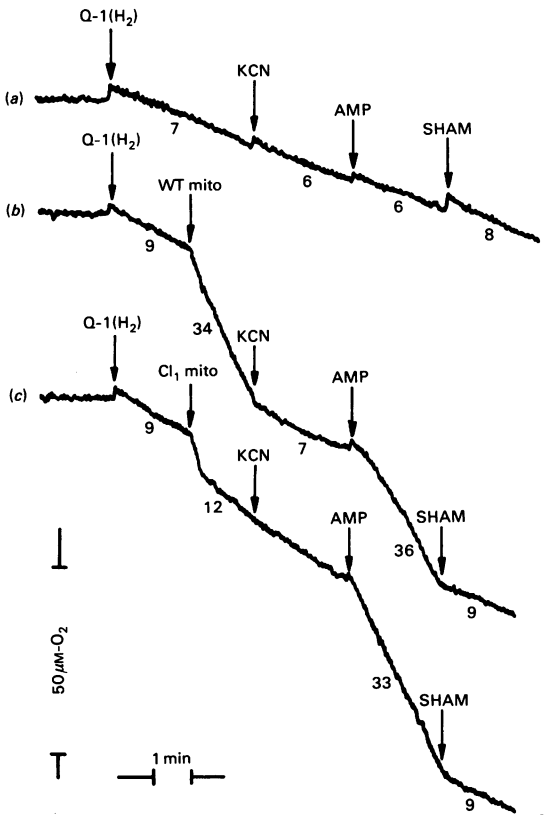

Fig. 6. *Oxidation of ubiquinol-1 by Paramecium mitochondria*

The medium used (1.5ml) is described in the Materials and methods section. The additions were at the following final concentrations: KCN, 1mM; AMP, 1mM; salicylhydroxamic acid (SHAM), 1mM; ubiquinol-1 [Q-1(H_2)], 300μM; mitochondria (mito), 1mg of protein. In the absence of mitochondria, ubiquinol-1 is spontaneously oxidized, and this autoxidation is not modified by AMP or salicylhydroxamic acid (trace *a*). Trace (*b*) corresponds to the oxidation of ubiquinol-1 by mitochondria from the wild-type (WT), and trace (*c*) to the oxidation of ubiquinol-1 by mitochondria from the mutant Cl_1.

cause of its spectral interference with ubiquinol-1. Because of this spectral interference, an indirect assay based on oxygraphy was carried out (not shown). A minute amount of the LAPAO extract of *Paramecium* mitochondria was sonicated with submitochondrial particles from ox heart. The oxygraphic assay revealed a cyanide-resistant respiration with pyruvate + malate, or succinate; this respiration was inhibited by salicylhydroxamic acid and stimulated by AMP.

It is noteworthy that extraction of quinol oxidase from *Paramecium* mitochondria by deoxycholate failed, in contrast with the report that deoxycholate readily extracts quinol oxidase activity from *Arum maculum* (Rich, 1978).

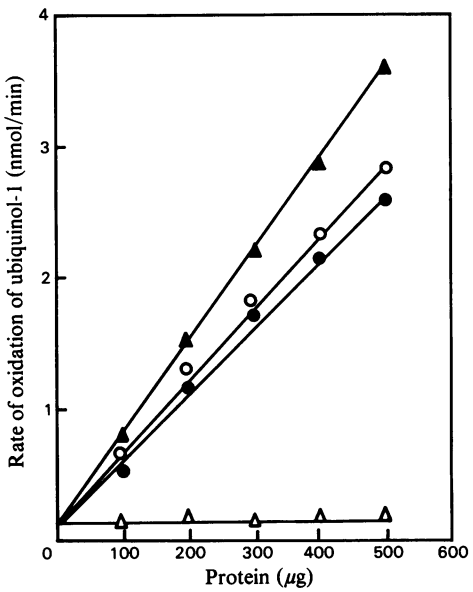

Fig. 7. *Oxidation of ubiquinol-1 by an extract of Paramecium mitochondria*

An extract of *Paramecium* mitochondria in LAPAO was prepared as described in the Materials and methods section (fraction S2). The proteins in the extract were denaturated by heating at 100°C for 3min. The final concentrations of KCN and AMP, where added, were each 1mM. The rate of oxidation of ubiquinol-1 was measured as described in the Materials and methods section. Key: ○, no extra addition; ●, KCN added; ▲, KCN and AMP added; △, heat-treated preparation. Salicylhydroxamic acid was not tested because of spectral interference with ubiquinol-1.

Discussion

Although the AMP-dependent stimulation of the cyanide-insensitive respiration is a well-established phenomenon for mitochondria of a number of microorganisms, including *Euglena gracilis* (Sharpless & Butow, 1970), *Paramecium tetraurelia* (Doussière *et al.*, 1979*b*), *Moniliella tomentosa* (Hansens & Verachtert, 1976; Vanderleyden *et al.*, 1979, 1980*a*), *Acanthamoeba castellanii* (Edwards & Lloyd, 1978) and *Neurospora crassa* (Vanderleyden *et al.*, 1980*b*), its mechanism has so far remained elusive. In this context, it was found that autoxidation of quinols, such as menadiol and duroquinol, is strongly enhanced by AMP, ADP and ATP (Vanderleyden *et al.*, 1980*b*); this was explained by a charge transfer between the quinol ring and the adenine ring of the nucleotides.

In the specific case of *Paramecium* mitochondria the following results are strongly indicative of an interaction between AMP and a protein component of the cyanide-insensitive respiratory path-

way. (1) Ubiquinol-1 added to *Paramecium* mitochondria is oxidized by these mitochondria in the presence of cyanide; the cyanide-insensitive quinol oxidase that is extracted from *Paramecium* mitochondria is activated by AMP. The protein nature of this quinol oxidase is suggested by the fact that the activity was destroyed by heat treatment. On the other hand, autoxidation of ubiquinol-1 at the pH of the test, 7.2, was not important and did not respond to AMP. (2) The photoactivable derivative of AMP, NAP_4-AMP, added to *Paramecium* mitochondria in the absence of light, was found to inhibit competitively the AMP-dependent respiration at micromolar concentration. The ratio K_m (AMP)/K_i (NAP_4-AMP) was close to 600. The very high affinity of NAP_4-AMP for the AMP site, compared with that of AMP, was unexpected, since NAP_4 has no effect as such on the cyanide-insensitive respiration. Such a high affinity is nevertheless indicative of specific interaction. (3) Photolabelling with NAP_4-AMP inhibited irreversibly the AMP-dependent respiration, but not the ADP-dependent respiration. Photoinactivation restricted to the AMP-stimulated respiration points to a specific protein component as target site for NAP_4-AMP. (4) Possible implication of bivalent cations brought by AMP is is ruled out by the finding that a similar stimulatory effect of AMP on respiration was obtained in an EDTA medium. Should metal ions be involved in cyanide-insensitive respiration, one would expect a better effect with ADP, which is a stronger chelator of bivalent cations than AMP. The experiments described in the present paper show that, in a carboxyatractyloside-supplemented medium, AMP stimulates the cyanide-insensitive respiration, whereas ADP is ineffective.

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