THE ACTION OF LIPOPHILIC ALKYLAMINES ON BEEF HEART MITOCHONDRIA

Evidence for the inhibition of proton translocation linked to electron transport

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1. Introduction

In a working hypothesis for the mechanism of electron transport phosphorylation it was proposed that in a lipophilic environment a proton-coupled ATP synthesis may involve the interaction of protein disulfide groups [1–3]. To trap the products of an assumed phosphorylytic cleavage of the disulfide function in a hydrophobic environment, namely a thiol and a sulfenyl group (RS^+) [1,2], lipophilic maleinimides [4], for the thiol group, and *N*-monoalkyl-thioureas [5], 6-alkyl-2-thiouracils [5], 1-alkyl-2-thioimidazoles [6] and 1-alkyl-2-thiobenzimidazoles [6], for the sulfenyl group, of increasing alkyl chainlengths were synthesized. In tightly-coupled beef heart mitochondria coupling was inhibited by each of the above thiol and sulfenyl reagents if their alkyl chainlengths were at least 9 carbon atoms long and if glutamate + malate were the substrates; these inhibitions could not be released by various uncouplers [2,7–9]. However, when succinate or ascorbate + TMPD was the substrate each of these lipophilic substances stimulated state 4 to state 3 respiration.

Even though the sulfur-free compounds of 6-nonyl-2-thiouracil and *N*-monononyl-thiourea were without effects in the above-described systems, as expected from chemical reasons [2], the corresponding sulfur-free compounds in the 1-alkyl-2-thio-imidazole and -benzimidazole series were much more effective in the inhibition reaction than the original thio compounds [9]. Because the 1-alkyl-imidazoles are stronger bases than the corresponding sulfur compounds [9], the inhibition of coupling and uncoupling observed, for example, with 1-decyl-imidazole or -benzimidazole, may be ascribed to the basic function of these compounds. These findings encouraged us to study the effects of unbranched primary alkylamines of increasing chainlength.

$$CH_3-(CH_2)_{n-1}-NH_2$$

on coupled respiration which is described in this report.

Abbreviations TMPD, tetramethyl-*p*-phenylendiamine, DNP, 2,4-dinitrophenol, FCCP, carbonylcyanide-*p*-trifluormethoxy-phenylhydrazone, SF 6874, 3,5-di-*tert* -butyl-4-hydroxy-benzylidenmalonitrile

[+]Part of the dissertation work

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2. Materials and methods

Isolation of mitochondria and measurement of respiration was as in [8].

2.1. *Determination of $\Delta\Psi$ and ΔpH of beef heart mitochondria after treatment with various concentrations of tetradecylamine in the presence of different substrates*

Beef heart mitochondria were prepared as in [8]. The incubation was carried out for 2 min with each of the substrates tested, as described in the legend of fig.4. The reaction was started by the addition of tetradecylamine and after 2 min terminated by centrifugation in an Eppendorf microcentrifuge for two minutes.

The matrix volume was determined with each of the substrates used by a double-labelling technique employing ^3H_2O and [^{14}C]sucrose, where ^3H_2O measures the total pellet water and [^{14}C]sucrose the extra matrix water [10]. The proton concentration gradient was determined by measuring the distribution of [^{14}C]acetate between supernatant and pellet fraction [10] for each substrate in the presence of increasing amounts of tetradecylamine. [^{3}H]Sucrose was added to each sample to follow in a double-labelling technique the changes in the extra matrix volume.

The membrane potential $\Delta\Psi$ was determined using ^{86}RbCl [11] and [^{3}H]sucrose. Radioactivity measurements were made in a Mark III liquid scintillation spectrometer (Searle Analytic Inc.) with the auto-isotope program no. 9. Corrections were made for changes in the matrix volume by assuming that it varied proportionally to changes in the extra matrix volume, the latter being determined as described above.

2.2. *Chemicals and reagents*

The sources of the reagents were Fluka (Buchs) heptyl-, nonyl-, decyl-, undecyl-, dodecyl-, tetradecyl- and hexadecylamine, puriss., Novo Industries, (Mainz) subtilisin Novo. [^{14}C]acetate, ^3H_2O and ^{86}RbCl were obtained from NEN, [^{3}H]sucrose and [^{14}C]sucrose from the Radiochemical Centre (Amersham). All the other reagents were obtained from Boehringer Mannheim.

3. Results

3.1. *The action of n-alkylamines with increasing chain-length on coupled respiration*

If the chainlength of the alkylamines was increased from the decyl to the dodecyl group or longer, a drastic change in the reactivity on coupled respiration of beef heart mitochondria was observed (fig.1). If glutamate + malate or β-hydroxybutyrate were used as substrates, coupled respiration was prevented by 80–140 nmol alkylamine ($R-NH_2$, R = $C_{11}H_{23}$ to $C_{16}H_{33}$), whereas 250 nmol decylamine/mg protein was required for inhibition. these were average values obtained from 10 mitochondrial preparations and these values decreased with increasing respiration control ratios. The inhibition reactions which were

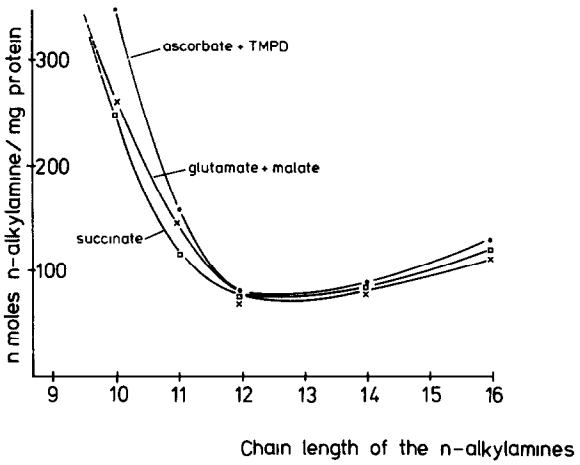


Fig.1. The concentrations of alkylamines required for either complete inhibition of coupled respiration with glutamate + malate (X) or ascorbate + TMPD (○) as the substrates or for complete stimulation of state 4 to state 3 respiration with succinate (□) The conditions are the same as in fig 2

accompanied by a 10–35% stimulation of state 4 respiration, could not be released by the uncouplers DNP, FCCP, or the most potent one, SF 6874 [12] Of the alkylamines of shorter chainlength only the heptyl- and nonyl compounds were able to inhibit state 3 respiration, a 30–50% inhibition required 350 nmol/mg protein.

With ascorbate + TMPD as the substrate a similar inhibition of coupled respiration was detected using similar concentrations of the individual alkylamines (fig.1) However, in contrast to the experiments with glutamate + malate, some inhibition of state 4 respiration was found concomitantly with the inhibition of coupling, the inhibition increased from 0–23% along the series hexadecylamine to decylamine. Again, the inhibition of coupled respiration could not be released by the uncouplers DNP, FCCP or SF 6847 [12].

When succinate was used as a substrate, however, state 4 respiration was stimulated completely to state 3 respiration by 50–120 nmol alkylamine ($R-NH_2$, R = $C_{11}H_{23}$ to $C_{16}H_{33}$)/mg protein (fig.1) Decylamine, however, enhanced the rate of state 4 respiration to only 40% of the state 3 respiration rate with 300 nmol/mg protein and also prevented coupled respiration

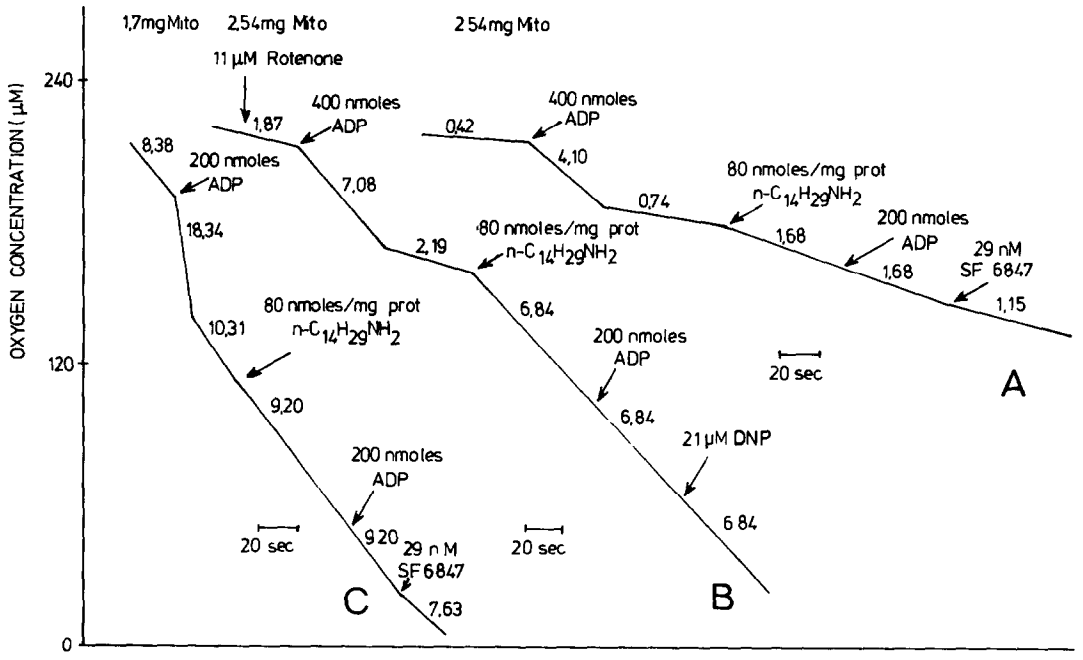


Fig. 2 Effect of tetradecylamine on coupled respiration of beef heart mitochondria The traces represent the output from an oxygen electrode The numbers on the traces are respiration rates, µmol oxygen mg protein^{-1} h^{-1} at 25°C Expt A Beef heart mitochondria (2.54 mg) were added to 2 4 ml of a reaction mixture which contained of 0 25 M sucrose, 2.5 mM glutamate, 2 5 mM D,L-malate, 5 mM malonate, 20 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate and 20 mM Tris·HCl (pH 7 3), Expt B Beef heart mitochondria (2 54 mg) were added to 2 4 ml of a reaction mixture consisting of 0 25 mM sucrose, 10 mM succinate, 20 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate and 20 mM Tris HCl (pH 7 3) Expt C Beef heart mitochondria (1.7 g) were added to 2.4 ml of a reaction mixture containing 0.25 M sucrose, 5 mM ascorbate, 0 25 mM TMPD, 20 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate and 20 mM Tris HCl (pH 7 3)

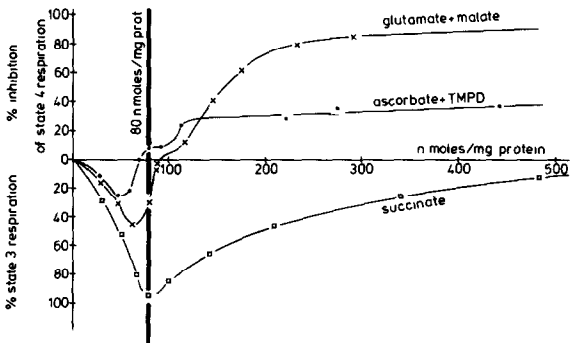
3.2. *The action of tetradecylamine on state 3 and state 4 respiration*

A minimum concentration of about 80 nmol tetradecylamine [CH_3–$(CH_2)_{13}$–NH_2] mg protein was found to inhibit coupled respiration in the presence of different substrates (fig.2). The titration curves of tetradecylamine concentration versus inhibition and stimulation of state 4 respiration are presented for each of the substrates in fig.3. The most striking result was that state 4 respiration with ascorbate + TMPD as the substrate could not be inhibited more than 35% by 450 nmol amine/mg protein, with glutamate + malate, however, state 4 respiration could be inhibited 90% by 300 nmol amine/mg protein (fig.3).

Fig.3 The action of tetradecylamine on state 4 respiration The conditions are the same as in fig 2.

3.3. *The effect of increasing concentrations of* n-*tetradecylamine on ΔpH and ΔΨ of steady state mitochondria with different substrates*

The changes of the membrane potential ΔΨ and the proton gradient ΔpH were determined in state 4



mitochondria with increasing concentrations of the amine present. Around the value of 80 nmol amine/mg protein, ΔpH and ΔΨ decreased with different ratios for each of the substrates used. With ascorbate as the substrate ΔpH diminished first, followed by ΔΨ both in an apparently 2-step break down. Surprisingly, ΔpH and ΔΨ broke down in very similar manner with glutamate + malate and with succinate as substrates.

3.4. *The effects on mitochondrial respiration of picric acid incubated together with, or after, tetradecylamine addition*

Picric acid was introduced [13,14] to facilitate the penetration of lipophilic cations through the mitochondrial inner membrane by the formation of a lipophilic ion pair. Inhibited coupled respiration, resulting from the preincubation of mitochondria with tetradecylamine, could not be released by equimolar amounts of picric acid (80 nmol/mg protein). This result was obtained with either ascorbate + TMPD or glutamate + malate as the substrates. When picric acid and the amine were mixed together in ethanol and added to state 4 mitochondria, respiration was stimulated to 50% of state 3 respiration with glutamate + malate as the substrate or to 160% of state 3 respiration with ascorbate + TMPD as the substrate. The latter experiments were carried out in the presence of antimycin A.

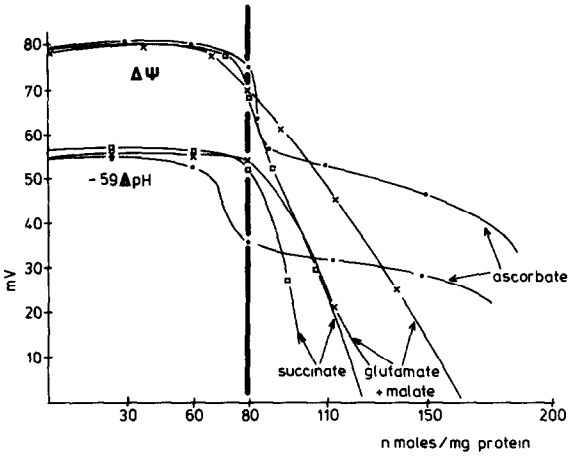


Fig.4. The changes of the proton concentration gradient ΔpH and the membrane potential ΔΨ in steady state beef heart mitochondria by increasing amounts of tetradecylamine in the presence of the different substrates The matrix volume was determined with ^3H_2O and [^{14}C]sucrose as in [10], the medium contained 2.11 ml 0.2 M sucrose, 10 mM Tris HCl (pH 7 2), 2 7 mg protein and 1 mM of the respective substrate(s). ΔpH was determined by measuring the distributions of [^{14}C]acetate as in [11], [^{3}H]sucrose was added to each sample to follow the changes in the extramatrix volume, the medium contained 2 16 ml 0.2 M sucrose, 10 mM Tris HCl, 25 mM Tris-acetate (pH 7.2), 2.7 mg protein and 1 mM of the respective substrate(s) ΔΨ was determined by a similar procedure using ^{86}RbCl and [^{3}H]sucrose (Mark III liquid scintillation spectrometer autoisotope program no 9), the medium contained 2 18 ml 0 2 M sucrose, 10 mM Tris HCl (pH 7.2), 10 mM RbCl, 20 nM valinomycin, 2.7 mg protein and 1 mM of the respective substrate(s) (K-salts) Each sample was incubated for 2 min with the respective substrate(s), the reaction was started by the addition of *n*-tetradecylamine and terminated after 2 min by centrifugation

4. Discussion

Lipophilic alkylamines were reported to be uncouplers of oxidative phosphorylation in mitochondria [15,16] and chloroplasts [17], furthermore lipid soluble proton- or hydroxylion-conducting agents were said 'in general and most unequivocally to short-circuit both phosporylation systems' [18]. We found, however, that unbranched primary alkylamines inhibited coupled respiration, if glutamate + malate or ascorbate + TMPD were the substrates and if the alkyl chainlength of the amine was at least 11 carbon atoms long (fig.1). An exclusively lipophilic interaction could be excluded because the corresponding hydrocarbons, e.g., tetradecane, did not effect these inhibition reactions. Furthermore, this inhibition could not be released by various uncouplers (fig.2). It was therefore very probable that the longchain alkylamines were acting near to the electron transport chain. The combination of basic function with a lipophilic chain in the alkylamines suggest that proton translocation is inhibited rather than electron transport. This idea is supported by the finding that state 4 respiration can only be inhibited to a maximum of 35% by high amounts of the amine in the presence of ascorbate + TMPD (fig.3). Therefore, it is very unlikely that the inhibition of coupled respiration is a consequence of the direct inhibition of electron transport. More precise information about the action of the amines was obtained by measuring changes of the membrane potential ΔΨ and the proton gradient

ΔpH in steady state mitochondria (fig.4). If the inhibitory concentration of tetradecylamine was exceeded, $\Delta\Psi$ and ΔpH decreased with each of the substrates used. Thus, the specific inhibition of the proton translocating units, as pumps [19] or channels, by the inhibitory concentration, is a reasonable explanation for both, the maintenance of $\Delta\Psi$ and ΔpH and the inhibition of coupling and uncoupling [21]. We are following the working hypothesis that the alkyl amines are tightly bound to the proton translocating units by both hydrophobic interactions and by an ionic linkage, the latter formed by the acid–base reaction of an undissociated carboxyl group of the proton translocating machine with the amine. This idea is further supported by the action of picric acid (section 3.4) which does not reverse the alkyl amine inhibition by subsequent addition, however, when the acid and amine are added simultaneously, the mitochondria became uncoupled. We do not, however, exclude the possibility that the requirement of a minimal chainlength of 11 carbons for the specific inhibition may be also taken as evidence for the hypothesis [20] of localized proton buried in the membrane. Concerning the unexpected stimulation of state 4 respiration with succinate as the substrate, we assume that the inhibition reaction was superposed by the probably general action of lipophilic substances as 'stimulating agents' at site II, the subject of further study.

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