THE ACTION OF LIPOPHILIC MALEIMIDES IN MITOCHONDRIAL ENERGY TRANSDUCTION

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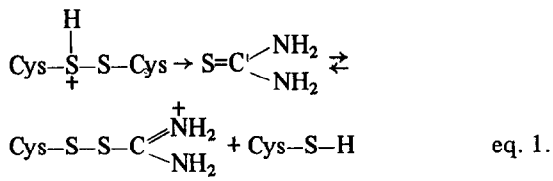
1. Introduction

We reported recently that lipophilic thiourea as well as thiouracils inhibit oxidative phosphorylation with glutamate + malate as substrates [1]. Thiourea cleaves cystine in strongly acidic solution [2] as follows:



eq. 1.

Therefore the above mentioned inhibition can be related to a protonized disulfide, which reacts like an activated thioester of the sulfenic acid (R–S–OH). It may be a functional group for an energy transfer in mitochondria and a mechanistic linkage to a proton-driven ATP synthesis [1]. With the formation of a proton-induced sulfenyl group (RS^+) a thiol group is liberated (eq. 1). The sulfenyl groups can be specifically and very rapidly trapped by thioureas and thiouracils [3].

*Part of the dissertation work

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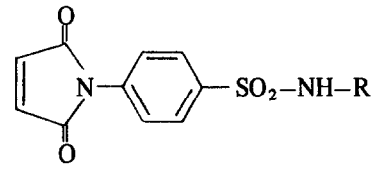
Abbreviations: ASPM, *N'*-(*N''*-acetyl-4-sulfamoylphenyl)-maleimide; NEM, *N*-ethyl-maleimide; NNM, *N*-*n*-nonyl-maleimide; NSPM, *N'*-(*N''*-nonyl-4-sulfamoylphenyl)-maleimide; NSPS, *N'*-(*N''*-nonyl-4-sulfamoylphenyl)-succinimide; DNP, 2,4-dinitrophenol; TMPD, tetramethyl-*p*-phenylenediamine; DIC, dicoumarol; MNT, mono-*n*-nonyl-2-thiourea; NTU, 6-*n*-nonyl-2-thiouracil; CCCP, *m*-chlorocarbonyl-cyanidephenylhydrazone.

To test our hypothesis in relation to the thiol group, we synthesized lipophilic maleimides (I) to combine lipophilic binding and chemical reactivity [4]. This report describes some properties of *N'*-(*N''*-alkyl-4-sulfamoylphenyl)-maleimides, of which the *n*-nonyl compound NSPM (Ia, R = C_9H_{19})



(I)

is a very potent inhibitor of oxidative phosphorylation and compares it with some other N-substituted maleimides [5].

2. Materials and methods

2.1. *Isolation of mitochondria*

Intact ox heart mitochondria were isolated according to the method of A. L. Smith [6], modified by the use of subtilisin. The mitochondria were separated into light and heavy layer fractions. The protein concentration was determined by the biuret method in the presence of 0.33% desoxycholate [7].

2.2. *Measurement of respiration*

Oxygen uptake was measured with a Clark-type oxygen electrode. All mitochondrial preparations were checked for structural integrity using the criterion of respiratory control [8].

2.3. *Chemicals and reagents*

N'-(*N''*-alkyl-4-sulfamoylphenyl)-maleimides were

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synthesized according to the ASPM-preparation (Ib, R = CH_3CO) of Merz et al. [9], but with an essential variation to avoid *N''*-acetylation [5]. In an analogous manner *N'*-(*N''*-nonyl-4-sulfamoylphenyl)-succinimide (II) was synthesized. *N-n*-nonyl-maleimide (III) was synthesized according to Heitz et al. [10]. The sources of other reagents were: Schuchardt, dicoumarol; Sigma, rotenone, NEM (IV); Novo Industries, Mainz, subtilisin, all other reagents were obtained from Boehringer, Mannheim.

3. Results

3.1. *The effects of N'-(N''-alkyl-4-sulfamoylphenyl)-maleimides (I) on the coupled respiration of mitochondria*

The lipophilic thiol reagent NSPM (Ia, R = C_9H_{19}) inhibits state 4 → state 3 transition of ox heart mitochondria using 12–16 nmol/mg protein and with glutamate + malate as substrates (fig.1). Only 8 nmol are necessary after incubating for 5 min. DNP (12.5 μM) cannot relieve the inhibition. If 90–110 nmol NSPM/mg protein are added, respiration is inhibited as completely as by KCN (0.4 mM). State 4 is stimulated to 90% of state 3 respiration by 20–26 nmol NSPM/mg protein with succinate (fig.1), even in the presence of oligomycin (0.6 nmol/mg protein). The same stimulation is caused by 28 nmol NSPM/mg protein with ascorbate + TMPD (fig.1). Investigations with other derivatives of (I) with decreasing carbon chain lengths (R = C_7H_{15}, C_5H_{11}, *i*–C_3H_7, C_3H_7, CH_3, H) revealed that only the heptyl derivate has similiar effects with glutamate + malate or succinate. The pentyl derivative, however, inhibits state 4 → state 3 transition with succinate.

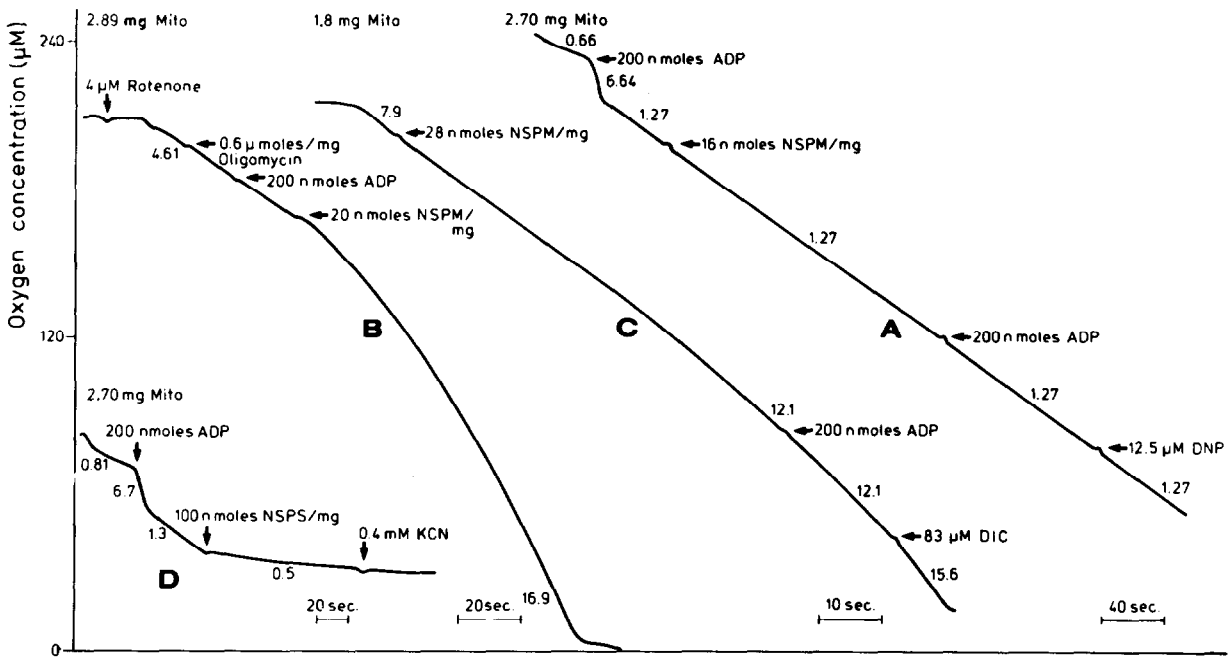
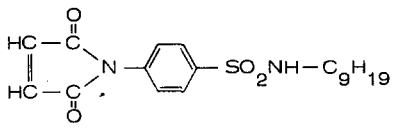
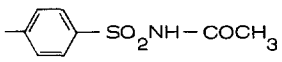
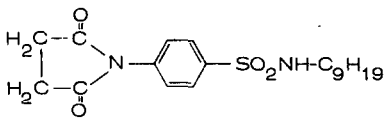


Fig.1. Effect of NSPM and the correlated succinimide NSPS, on the coupled respiration of ox heart mitochondria. The lines represent the output from an oxygen electrode. The numbers on the lines are respiration rates, μmoles of oxygen mg^{-1} protein h^{-1} at 25°C. Experiment A and D: ox heart mitochondria (2.70 mg) were added to a reaction mixture consisting of 2.4 ml 0.25 M sucrose containing 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 7.4. Experiment B: ox heart mitochondria (2.89 mg) were added to a reaction mixture consisting of 2.4 ml 0.25 mM sucrose containing 10 mM succinate, 20 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate and 20 mM Tris–HCl, pH 7.4. Experiment C: ox heart mitochondria (1.80 mg) were added to a reaction mixture consisting of 2.4 ml 0.25 M sucrose containing 2.5 mM ascorbate, 0.25 mM TMPD, 20 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate and 20 mM Tris–HCl, pH 7.4.

Table 1
Effects of various N-substituted maleimides on the respiration of ox heart mitochondria with glutamate + malate as substrates

No.	Formula	Abbr.	Inhibition of state 4 → state 3 transition (nmoles/mg protein)	Respiration inhibition (nmoles/mg protein)
Ia		NSPM	12 - 16	90 - 110
Ib		ASPM	without effect up to 50	110 after addition of ADP
III		NNM	60 +30 % stimulation of state 4 respiration	-
IV	$-C_2H_5$	NEM	without effect up to 50	200 (50 %)
II		NSPS	without effect up to 50	120

The conditions are the same as described in fig.1 for the experiments A and D. The concentrations indicate maximal inhibition, unless otherwise indicated. Maximal inhibition of respiration means the same degree of inhibition as with 0.6 mM KCN.

Table 2
Effects of various N-substituted maleimides on the respiration of ox heart mitochondria with succinate

No.	Abbreviation	uncoupling (nmoles/mg protein)	Inhibition of state 4 → state 3 transition (nmoles/mg protein)
Ia	NSPM	20–26	–
Ib	ASPM	no effect	110
III	NNM	120	–
IV	NEM	without effect up to 300	
II	NSPS	no effect	170 +50% stimulation of state 4 respiration

The conditions are the same as described in fig.1 for experiment B. The concentrations indicate either maximal uncoupling, i.e. state 3 respiration, or maximal respiration inhibition, i.e. inhibition as with 0.6 mM KCN.

3.2. *The effects of other N-substituted maleimides on the coupled respiration of mitochondria*

If the *n*-nonyl group is substituted by acetyl, this well-known ASPM (Ib, R = CH_3CO) is without effect up to 50 nmol/mg protein on the coupled respiration with glutamate + malate as substrates (table 1). But respiration is completely inhibited by 110 nmol/mg protein, only if ADP is added subsequently. In contrast to NSPM, state 4 → state 3 transition is inhibited by 110 nmol ASPM/mg protein with succinate (table 2) If the lipophile alkyl ligand is combined directly with maleimide in *N*-(*n*-nonyl)-maleimide (III, NNM), 60 nmol NNM/mg protein inhibit state 3 respiration with glutamate + malate, accompanied by some stimulation of state 4 respiration. The latter is stimulated with succinate to 80% of state 3 respiration by 120 nmol NNM/mg protein. The reduction of the alkyl chain to an ethyl group in NEM (IV) caused this maleimide to be effective as respiration inhibitor only in high concentrations with glutamate + malate as substrates (table 1); with succinate no change in respiration and ATP formation can be detected.

3.3. *The effects of the correlated succinimide (II) to the lipophilic maleimide NSPM (Ia) on the coupled respiration*

To distinguish between chemical reaction and lipophilic interaction, the saturated analogue of NSPM, the *N'*-(*N''*-nonyl-4-sulfamoylphenyl)-succinimide (II, NSPS) which cannot add a thiol group, was introduced. With glutamate + malate as substrates 120 nmol NSPS/mg protein inhibits respiration as well as KCN (fig.1). With succinate 170 nmol NSPS/mg protein prevents state 4 → state 3 transition accompanied by 50% stimulation of state 4 respiration (table 2).

4. Discussion

The lipophilic thiol reagent NSPM (Ia) with a C_9-alkyl chain shows the same effects as the corresponding lipophilic trapping agents for sulfenyl groups (RS^+), the mono-nonyl-thiourea (MNT) and the 6-nonyl-2-thiouracil (NTU) [1]. With glutamate + malate as substrates oxidative phosphorylation is inhibited, with succinate state 4 respiration is stimulated, both by as little as 1/10 of the MNT and NTU concentrations. The heptyl derivative is less effective, whereas the shortened alkyl chain in the pentyl derivative causes a complete change in reactivity in direction to ASPM (Ib, table 2).

The inhibition of electron transport using a sevenfold concentration of NSPM and glutamate + malate as substrates (table 1) suggests a two step reaction of NSPM. Because of the reaction of its saturated analogue NSPS (II), which inhibits respiration with nearly the same concentration (table 1), this second step can be related to the lipophilic interaction. The first step with low concentrations of NSPM (table 1) is the chemical reaction where the thiol group is essential for the energy transduction in mitochondria. Uncouplers such as DNP or CCCP cannot release this inhibition of state 4 → state 3 transition and therefore from a comparison with DCCD the reaction of NSPM is thought to be between the electron transport chain and ATP synthesis, probably nearer to the former.

The very similar effects of the thiol reagent NSPM and the sulfenyl group reagents MNT and NTU support the idea that an activated disulfide (eq. 1) may be involved in the energy transduction of mitochondria, probably near to the Fe–S centers of complex I.

The very specific action of NSPM is not only a function of the alkyl chain, but also of the benzene ring. This is shown by comparison of maleimides with the C_9-alkyl chain alone (NNM) and with the benzene ring plus an acetyl group (ASPM) (table 1). The covalentyl labelled proteins will be identified using [^{14}C]NSPM in further investigations.

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