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THE EFFECT OF THE LIPOPHILIC THIOL REAGENT NSPM ON OX.PHOS. AND RESP.CHAIN

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The lipophilic thiol reagent NSPM (N'-[N''-n-nonyl-4sulfamoylphenyl]-maleimide) has been introduced as trapping agent for thiol groups in hydrophobic environment [l]. Now it will be demonstrated that NSPM acts not only as thiol trapping agent, but also interacts non covalently with high affinity binding sites and abolishes very effectively important mitochondrial functions. In addition reaction as acylating reagent is possible.

1) NSPM inhibits the respiratory chain in the span from NADH to Coenzyme Q, but does not inhibit the oxidation of NADH by Ferricyanide or Menadione (respiratory chain inhibition on mitochondria at 100 nmol/mg, on submitochondria at 20 nmol/mg). Labeling of ETP_H/CI with ^{14}C-NSPM shows that there is no correlation of inhibition with covalent modification of thiol groups. A comparison of results obtained with the structural similar non-thiol-reagent ANSA (N'-Acetyl-N'-n-nonylsulphanilamide) confirms these data, because similar to NSPM, ANSA is a very potent site I specific respiratory chain inhibitor (mito.resp. chain inhib. at 75 nmol/mg, ETP_H at 10 nmol/mg). ANSA has no other effects as NSPM has.

2) NSPM inhibits the ß-hydroxybutyrate-DH activity. There is a close relationship between inhibition and radioactivity labeling of the 33 000 M_r-DH(max. inhib. on ETP_H with 25 nmol/mg). The labeling is sensitive to Diamide treatment [2].

3) NSPM binds to the Nucleotide Carrier. The binding is sensitive to CAT [2].

4) NSPM acts like an uncoupler. It inhibits ox.phos. (60 nmol/mg), ATP-Pi-exchange (60 nmol/mg) and energy linked transhydrogenase, it stimulates the ATPase (450 nmoles/mg) and the proton release of submitochondrial particles:

a) For the ATP driven transhydrogenase is in the presence of KCN instead of Rotenone more inhibitor needed (130 nmol/mg instead of 70 nmol/mg) and the inhibition is time dependent. The non-energy linked transhydrogenase is blocked at about 450 nmol/mg.

b) The proton release shows a biphasic behavior (t1/2 decreased by a factor of 7 at 60 nmol/mg). NSPM competes with NPA on the high and low affinity binding sites for uncoupler (in mitochondria) and does not compete with DCCD-binding (on complex V).

From a comparison with structurally similar compounds it may be concluded that NSPM acts not as a simple "protonophoric" compound. Since the uncoupling seen on mitochondria with succinate as substrate is time dependent (2-3 minutes until maximal stimulation) the effect could be excerted by other effects, i.e. chemical reactions.

5) In the isolated OS-ATPase NSPM inhibits the ATP-P_i-exchange activity, as in submitochondria, very effectively. NEM has not effect at the same concentration. There is no correlation in labeling by ^{14}C-NSPM with the inhibition. The radioactivity of the dialyzable band at the dye front increases with -NSPM conc. [3]. The inhibition of the ATPase at higher concentrations of NSPM or longer preincubation can be correlated with the extent of labeling of the α -band by ^{14}C-NSPM. Extrapolation to 100% inhibition gives about 1.7 nmol ^{14}C-NSPM/mg ATPase, which suggests 1 nmol NSPM/nmol ATPase is enough for 100% inhibition.

From the above results it may be suggested that NSPM has a preference for nucleotide binding sites, either with or without trapping thiols (or other groups by acylation).
Since in all studies with thiol reagents, there is no correlation between inhibition/uncoupling of energy-linked reactions on mitochondria/submitochondria or complex V with incorporation of radioactivity into a thiol containing factor [as described in 4,5] it may be concluded that inactivation of isolated F_B with thiol reagents [4,5] is unrelated to the effects of these reagents on energy transducing particles or isolated ATP-P_i exchange catalyzing complex.

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ON THE Ca^{2+}-PHOSPHATE SYMPORTER

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250 μM picrylacetate (PA) has been reported to uncouple oxidative phosphoryl. and inhibit partly respiration in rat liver mitochondria [l]. This effect has been ascribed to picrid acid liberated inside the mitochondria. Picrid acid as such has no effect on mitochondria [1,2] but in submitochondria inhibits ox.phos. causing only a small increase in proton permeability if compared with DNP [2].

From the chemist's point of view, PA is a highly reactive acetylating reagent rather than a picrate-generating system. For this reason the effect of the compound on mitochondria was reinvestigated.

30-35 nmol PA/mg protein (13-15 μm) inhibit state 4 - state 3 transition of rat liver mitochondria at sites I-III and II-III to more than 90%. Inhibition of state 4 - state 3 transition with ß-hydroxybutyrate es substrate cannot be observed because of a concomitant inhibition of respiration at 6-10 nmol PA/mg protein (3-5 μM).

The effects on ox.phos. and resp. chain are not reversed by addition of BSA. However, a slight stimulation of state 4 by PA is reversed by BSA-treatment. The inhibition of state 4 - state 3 transition is released to about 50 % by uncouplers and reversed by DTE (15 mM) to about the same extent.

In the presence of 30 to 40 μM PA the mitochondrial respiration rate is above state 4 level, which cannot be increased by uncouplers.

Arsenate (1.7 mM) and Ca^{2+} (1.7 mM)-stimulated respiration are inhibited by 13-15 μM PA, which therefore acts differently than the inhibitors carboxyactractyloside and Oligomycin.

0.57mM Ca^{2+} in presence of 10 mM succinate induce a respiratory burst down to $0_2 = 0$ if enough phosphate is present. The minimal concentration of phosphate is about 1.2 mM. In the presence of smaller concentrations such as 0.6 mM phosphate the increased respiratory activity

declines after a certain time. It can be restored by addition of more phosphate. Similar results were obtained for arsenate.

The addition of 13-15 μM PA shortly (~ 2 sec) after addition of Ca^{2+} in the presence of phosphate (or arsenate, > 1.2 mM) inhibits the increased resp.rate after a lag of about 15 seconds. After further 50 more seconds the respiration starts to increase again to about 35% of the initial Ca2+ induced respiratory rate. The lag of 50 sec. can be abolished by addition of uncouplers at the inhibited state.

The ATPase of whole mitochondria is stimulated with 40 μM PA to a maximum of 5 times the original ATPase (0.1 μM ATP/min mg).

Oxygen pulse experiments on whole mitochondria (succinate as substrate) show that a maximum of inhibition of t1/2 of H+-reentry (by a factor of 1.4-1-5) is obtained with 40-50 μM PA. At 200 μM PA there is a time dependent stimulation of H+-reentry by a factor of 6-7. Stimulation of ATPase and inhibition of t1/2 are parallel effects and occur at the same conc. of PA which keep respiration above state 4 level. In the presence of submitochondria PA is hydrolyzed rapidly, and the studies done on mitochondria are not possible. At high PA concentration, where similar conc. of picric acid stimulates ATPase, the ATPase activity is also increased.

The result are compatible with the following conclusions:

1) > 200 μM PA: uncoupling as described in [l] by picric acid released inside mitochondria.
2) 40-50 μM PA: inhibition at state 4 by blocking the uncoupler binding site with picric acid [2] and acetylating nucleophilic group(s) in the ATP synthase resulting in ATPase conformation.
3) 13-15 μM PA: inhibition of state 4 - state 3 transition by blocking the phosphate translocator by acetylating nucleophilic group(s) involved in translocation. Suggested is reaction on a Ca^{2+} -phosphate-symporter similar to the one postulated by Mitchell [3 , 4].
4) 3-5 μM PA: inhibition of ß-hydroxybutyrate-DH by acetylating this enzyme.

Preliminary labeling experiments with 3H-PA showed labeling of bands at 10000, 33000 (Boxy-DH), 120000 and 150000 Mr on SDS-gels in the absence of mercaptoethanol. Further labeling experiments are in progress.

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ISOLATION AND IDENTIFICATION OF MITOCHONDRIAL UNCOUPLER BINDING PROTEINS.

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Among the proteins implicated in the mitochondrial uncoupler binding site [l] are subunit 1 of Fl-ATPase [2] and an otherwise uncharacterized protein band at araund 30 000 daltons (30 kD) detected by covalent labeling with radioactive 2-azido-nitrophenol (NPA), a light-sensitive

uncoupler [3]. Earlier, it was found that purification of the nucleotide carrier [4] from NPA-labeled mitochondria resulted in a preparation essentially free of NPA. The nucleotide carrier was therefore believed not to contribute to the NPA labeled band around 30 kD [2].

We now report that in the presence of protease inhibitors and mercaptoethanol, three NPA-labeled polypeptides can be identified in the Mr range of 29-33 kD. In hydroxyapatite chromatography [4], the first labeled component apears in the pass-through fraction, together with the nucleotide carrier. The other two polypeptides (Mr ~32 kD, 29 kD) are eluted with a shallow Pi gradient in two partly overlapping peaks. The extent of labeling in the nucleotide carrier is about as much as in the combined two other peaks. Another polypeptide (55 kD, probably subunit 1 of F_1 elutes at höher Pi concentration and comprises about 20% of the protein-bound radioactivity. The possible involvement of proteolysis was tested by adding purified NPA-labeled carrier to unlabeled mitochondria during solubilization in the absence of protease inhibitors and carboxyatractylate (CAT). From this mixture, no radioaktive component in the 30 kD region was eluted with the Pi gradient. In a control experiment in the presence of protease inhitbitors and CAT, the NPA-labeled carrier was the only radioaktive component recovered. Amino acid analysis of the 29 kD band in the Pi-eluted peak indicated that this polypeptide is not related to either the nucleotide carrier, subunit 1 of Fl, or Factor B.

The participation of 30 kD polypeptides in specific uncoupler binding was studied by competition experiments. Beef heart mitochondria were photo-labeled with NPA, NPA and DNP (a competitive-inhibitor of specific equilibrium binding of NPA, ref.1), and NPA + picrate (not-a competitive inhibitor, ref. 5). DNP inhibited drastically (~14 fold) labeling of subunit 1 of F_1 while the 30 kD band was only diminished by a factor of 3. In contrast, picrate decreased both bands 4.6- and 3-fold, respectively, probably mainly through a filter effect. Thus, subunit 1 of Fl appears to be a component of the high affinity, specific uncoupler binding site. The above data seem to exclude the nucleotide carrier as another component of this site, but not necessarily the other two components in the 30 kD region.

While the role of the uncoupler binding site is not clear [6], new data concerning the function of 30 kD proteins are available [7]. An ATPase preparation similar to complex V [8] has been abtained which is devoid of NPA-binding 30 kD proteins. This preparation shows high ATP-dependent oxonol response [9], good ATP-P_i exchange activity and full oligomycin sensitivity. It differs from complex V by being sensitive towards freezing and by a diminished DCCD-sensitivity. These data suggest that one (or more) of the NPA-binding 30 kD proteins is an integral part of a stable ATPase complex, without directly participating in energy dependent reactions confined to the complex.

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INTERACTION OF PICRATE AND PICRYLACETATE WITH THE MITOCHONDRIAL ENERGY TRANSFER AND UTILIZATION SYSTEM

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The high energy compound picrylacetate (PA) is not only a powerful acetylating reagent but also a selective one, probably by charge transfer interactions with aromatic amina acids. It appears to be able to trap functional groups involved in a chemical intermediate cascade from redox reaction to phosphorylation by functional group exchange or by transacetylation. Relevant data will be presented on experiments using rat liver mitochondria (RLM), beef heart submitochondria (SMP) and chloroform released ATPase (BF_1) derived from the latter. Some of the results on RLM have already been described [l].

1. RLM) At 13-15 μM PA, state 4 - 3 transition at sites I - III (45% reversed by DTE or released by DNP) and II-III (80% reversed by DTE or released by DNP) is inhibited, as is arsenate and Ca^{2+} -stimulated respiration (not reversed by DTE). However, $^{33}P_i/P_i$-exchange and Ca2+-content are not much affected. Considerable amounts of picrate are accumulated and ATPase is stimulated.

At 30-40 μM PA, respiration is at state 6 level (not DNPsensitive), ΔpH can still be established, and H+-reentry is inhibited ($-p_i$). $^{33}p_i$/Pi-exchange is about 50% inhibited, Ca2+-content is not much affected, but Ca2+ induced active swelling is abolished (DNP-sensitive). The ATPase is maximally stimulated (aligomycin-sensitive) and high amounts of picrate are accumulated (>100 μM). Picrate as such has no effect on RLM [2] .

Labeling of RLM (RCR >4, succ) with 40 μM H-PA gives incorporation of about 0,4 nmol 3H-acetyl/mg RLM into both a 30.000 and a 13.000 Mr band. The capacity of both bands is about 2.2 nmo1 3H-acetyl/mg RLM. The band at Mr ~13.000 is extractable with acid chloroform-methanol. Labeling of RLM (RCR <2, succ) incorporates radioactivity mainly into the extractable band.

2. SMP) The effects of 40 μM PA and picrate (table I) may be summarized as follows: ATP-Pi-exchange, reverse electron transfer and Δφ are abolished, ATPase is inhibited, and ΔpH is increased by PA. Picrate itself lowers ATP-Pi-exchange and Δφ, stimulates ATPase (opposite to PA inhibition) and has little effect on ΔpH.

Labeling of SMP with 40 μM 3H-PA gives incorporation of radioactivity into two 30.000 Mr bands as well as a 13.000 Mr band. There is incorporation of about 0.07 nmol 3H-acetyl/mg SMP into one of the 30.000 Mr bands and of 0.05 to 0.12 nmol 3H-acetyl/mg SMP each into a 30.000 Mr band and ~13,000 Mr band, depending on the conditions used (Mg-succ <Mg-ATP< Mg-ATP-Picrate).

Table I: SMP-activity		PA,40 µM -%	PA,40µM +BSA/%	picrate 40 µM/%	Cd2+ 25 µM/%
ΔpH (ACMA)	Succ	500-1200[a)]	-	no effect	10
	ATP	150	-	no effect	150
Δφ (OX-VI)	Succ	10	-	50	50
	ATP	15	-	60	100
ATP-Pi		10	35	35	100
ATPase		70[b)]	82	120[c)]	100
rev.e^-(+/-DTE)		5	22	82	100
Succ→O2		146	100	146	15
NADH→O2		50	35	120	100
Succ→Transh.		71	87	68	15
ATP→Transh.		63	72	87	100

a)the absolute value is similar to the value obtained with ATP; b) 50% ATPase at 160 µM PA; c) max. ATPase (155%) at 160 µM picrate.

By comparing the effects on RLM, SMP and BF_1, it can be concluded that PA, at low concentration, and picrate are site specific inhibitors. PA abolishes energy transfer from respiratory chain to ATP synthetase ('decouples') and energy utilization by the ATP-synthetase in SMP by acetylation (functional group exchange). Picrate (either by itself in SMP or supplied in RLM by PA reaction) inhibits energy utilization by turning ATPsynthetase into an ATPase. The site specific "uncoupler" Cd2+ [3] is shown as an agent acting on respiratory chain activity in SMP exclusively.

3. BF1) As in SMP, picrate stimulates and PA inhibits ATPase activity. The inhibition by PA in biphasic, the first phase being reversible with DTE. Acetylation of a ß-subunit tyrosin (transacetylation), sensitive toward nucleophiles (As_i, Pi, SO_3^{2-}, DTE, J-) and NBD-Cl, at the phosphate binding domain, is responsible for ATPase inhibition. Acetylation of α- and γ-subunit amino groups is sensitive toward ADP+Pi, aurovertin, NSPM.and cold treatment, of α alone toward pH, AMP-PNP and ADP. PA itself changes the sensitivity of the α-subunit toward 3H-NPA labeling. It is concluded that PA affects and probes subunit interactions (conformations) in BF_1.

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INTERACTION OF NITROPHENOLS WITH THE MITOCHONDRIAL ATPase

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It has long been known that uncouplers stimulate the mitochondrial ATPase in the membrane-bound and soluble form(F1).The present study focusses on the interaction of F1 with nitrophenols, nucleotides and the mitochondrial inhibitor protein (IF_1).

2-Azido-4-nitrophenol (NPA) stimulates the ATPase activity of Fl by 30%, and in submitochondrial particles by a factor of up to 2.5, depending on the method of particle preparation. In S-particles (where IF1 has been removed by Sephadex treatment), no such stimulation occurs. In these particles, incorporation of NPA into the ß subunits by photoaffinity labeling is up to two times higher than in untreated particles. Similarly, addition of IF1 to F1, in the presence of Mg-ATP decreases NPA-labeling in ß, but not α . These data indicate that IF1 inhibits the binding of nitrophenols, and suggest that uncouplers stimulate the ATPase by weakening the interaction between IF1 and Fl. This is in agreement with findings of others that i) high uncoupler concentrations solubilize the inhibitor protein [l] , ii) the ß subunit interacts with the inhibitor protein [2], and iii) in intact mitochondria, the α, but not the ß subunit is labeled by NPA [3].

With repeated cycles of photoaffinity labeling of Fl with NPA (including removal of non-covalently bound photo-products by the centrifuged column procedure [4]), it is possible to saturate NPA binding (Fig. 1). The number of binding sites is 5-6, the same as for nucleotides [4]. During this treatment, the ATPase activity does not become inhibited as compared to the control. The number of tightly bound nucleotides, however, decreases. Conversely, addition of Mg-ATP decreases the labeling of subunit ß, while the incorporation of NPA into subunit α remains unchanged.

These and the above data suggest interactions between the binding sites of nucleotides, nitrophenols and IF1 on the ß subunit of F1.

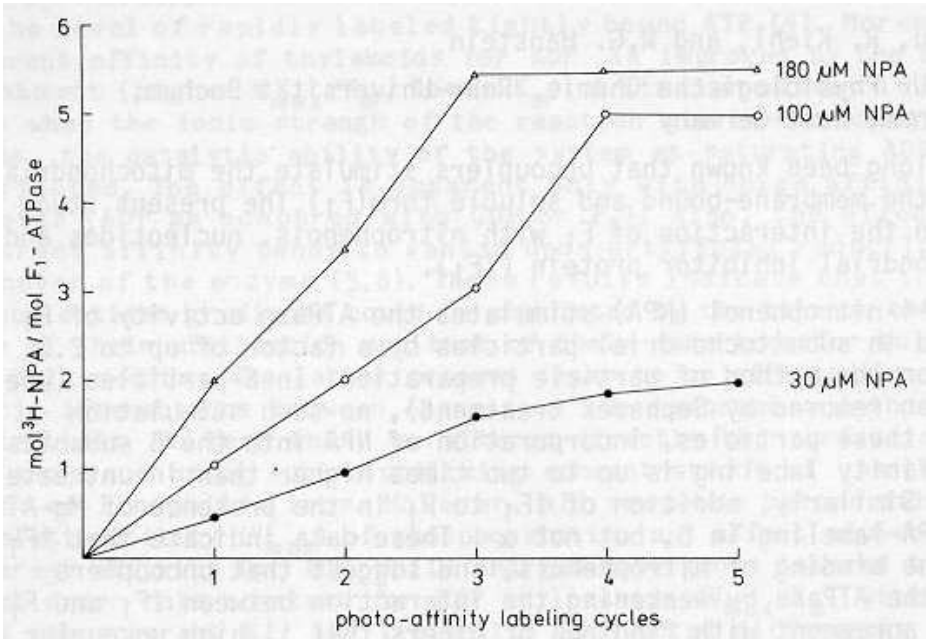

Fig. 1: Determination of the number of nitrophenol binding sites in Fil Repetitive photoaffinity labeling with the NPA anion. Each cycle includes photolabeling, removal of remaining NPA and its non-covalently bound photo products, and addition of the indicated concentrations of fresh NPA.

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CONFORMATIONAL STATES OF BHM-ATP SYNTHETASE AS PROBED BY THE POTENTIAL SENSITIVE DYE OXONOL VI

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Complex V has been isolated as the segment of the mitochondrial oxidative phosphorylation system which is concerned with ATP synthesis and hydrolysis. In the isolated state, complex V catalyzes, without reconstitution procedures, oligomycin-sensitive hydrolysis of ATP, oligomycin and uncoupler-sensitive ATP-Pi-exchange [l], shows oligomycin and uncoupler-sensitive energy dependent oxonol VI response but no energy dependent ACMA-quenching [2] even after removal of AmS04 from the ATPase complex. The preparation is reported to be essentially free of vesicularized structures in electron microscopic studies [l]. KSCN inhibits the dye response by about 10-27%, depending on the preparation, which may be an estimate of the amount of vesicularized structure present. The extent of the oxonol response depends on the ATP/ADP ratio rather than the phosphorylation potential. Studies on complex V activities suggest that oxonol VI reflects, at least in part, a more local, ATP-dependent and energy-related process [3]. The oxonol VI response may be used, therefore, as a probe for conformational states of the ATP synthetase, as demonstrated by the following results.

The nucleotides ITP, GTP and UTP are hydrolysed by the complex, but considerable NTP-Pi-exchange is observed only with ATP. ITP and GTP give rise to 50%, and UTP only to 25% dye response. An ATP concentration far below the K_m (~160 μM) for ATPase elicits also partial (about 50%) and declining dye response, which suggests that energy is required to maintain the response. 50 μM AMP-PNP prior to addition of 5 mM ATP inhibits ATPase activity to 90%, increases ATP-Pi-exchange and limits the dye response to about 50%. The dye response can be titrated with ATP in the presence of an ATP regenerating system and l0 mM Mg2+. There are four steps in the titration curve. Breaks appear at about 25, 50 and 75% dye response and at about 8, 45 and 140 μM ATP. 100% dye response is reached at about 300 μM ATP. This may be explained by negative cooperativity in four discrete ATP binding steps which induce progressive dye response. The response at low steady state ATP can be modulated by K+, Mg2+ or Ca2+-ions. K+ enhances the negative cooperativity and limits the dye response to 50% at 290 μM ATP (10 mM Mg2+ present). Low steady state ATP (100 μM) induces, in the absence of added Mg2+,

partial dye response. Subsequent addition of Mg2+ (1 mM) results then in almost 100% response.

The difference between the response at low and saturating Mg2+ concentration is about 50%. Ca2+ in the presence of saturating Mg2+ also lowers dye response.

The temperature dependence of the ATP induced oxonol response reveals a break at about 50%: at 10°C, there is rapid response to about 50% and much slower response to 100%.

Aurovertin, which has been used to study conformational changes in F1, inhibits ATPase to 90%, inhibits ATP-Pi-exchange to 97%, but inhibits dye response only to 50%. Addition of limited amounts of the nitrophenol compounds, HE-DNP, DNP, AE-DNP, picrate and NPA (causing 2 to 10% abolishment of dye response) results in cumulative abolishment of the response. No such effects are observed in the presence of oligomycin and uncouplers. The enhanced effects of the compounds may be explained with an aurovertin promoted conformation and an enhanced binding of nitrophenols.

The partial dye responses as described above may be explained assuming different conformational states of the complex. The progressive conformational changes induced by ATP are similar to the changes induced by NAD-binding to GPDH [4]. The multisubunit ATP synthetase may be a case in which the ligand induced changes show predominantly negative cooperativity, and where the half-of-the sites reactivity [5] may only be one extreme form.

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