DOES HYDROPHOBIC ISOTHIOCYANATE REALLY UNCOUPLE OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA?

Uncoupling activity of a product of isothiocyanate in dimethylsulfoxide solution

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1. Introduction

A wide variety of organic compounds are known to be uncouplers of oxidative phosphorylation in mitochondria [1]. Since uncouplers have a dissociable proton and act as proton conductors in mitochondria [2–4] and model membrane systems [5–7], their action is thought to be due to collapse of the proton gradient as a result of proton transfer across the mitochondrial membrane. Alternatively they may have a direct interaction with some mitochondrial protein involved in oxidative phosphorylation [8–10].

Hydrophobic isothiocyanates, such as BrPh-NCS, were reported [11] as effective uncouplers in mitochondria, stimulating the respiration of state 4 mitochondria, releasing oligomycin-inhibited respiration and activating ATPase, BrPh-NCS, the most effective isothiocyanate, stimulated state 4 respiration of rat liver mitochondria maximally at ~50 μM with succinate as substrate, and activated ATPase at ~15 μM [11]. If BrPh-NCS really uncouples oxidative phosphorylation in mitochondria, it would be the only known potent uncoupler that is not weakly acidic. In this case isothiocyanate would act as an SH-reagent directly reacting with an SH-group in mitochondrial protein as proposed [11].

However, as shown in [4], isothiocyanates are very reactive with non-proteinous –SH or $-NH_2$ groups; under mild conditions they form diffusible, weakly acidic dithiocarbamates with compounds having an –SH group and these uncouple oxidative phosphorylation probably by dissipation of the proton gradient. Thus, exact information on the mechanism of action of hydrophobic isothiocyanates, such as BrPh-NCS, is important for understanding the mechanism of oxidative phosphorylation as well as the mechanism of uncoupling in mitochondria.

This paper deals with the effect of BrPh-NCS on mitochondrial function, using DMSO as a solvent of the stock solution as in [11].

Abbreviations: BrPh-NCS, 4-bromophenylisothiocyanate; DMSO, dimethylsulfoxide; BBTU, *N,N'*-bis(4-bromophenyl)-thiourea

2. Materials and methods

BrPh-NCS was kindly supplied by Dr M. Miko, Slovak Polyt. Univ. and was also synthesized by the method in [12]. BBTU was synthesized from BrPh-NCS as follows: A solution of BrPh-NCS in DMSO was stood at room temperature for 3 days then diluted with water to obtain a solid which was recrystallized as needles from ethanol (m.p. 184–185°C). The IR and NMR spectra of the compound agreed with those of BBTU prepared by the method in [13]. Rotenone was a gift from Sumitomo Chemical Industry, Osaka and oligomycin and valinomycin were from Sigma Chemical Co., St Louis. ATP and ADP were obtained from Kyowa Hakko Co., Tokyo. Other reagents were standard commercial products and were used without further purification.

Rat liver mitochondria were isolated by the method in [14] as in [15].

The respiratory rate of mitochondria was measured with a Clark oxygen electrode, Yellow Spring Instruments, at 25°C in a medium consisting of 200 mM sucrose, 2 mM $MgCl_2$, 1 mM EDTA and 10 mM potassium phosphate (pH 7.4). The total volume of the reaction mixture was 4.35 ml.

ATPase activity was determined by measuring the pH change of the medium due to hydrolysis of ATP in mitochondria with a Hitachi-Horiba pH-meter, model F-7, according to the method in [16]. The medium consisted of 100 mM sucrose, 0.5 mM EDTA, 50 mM KCl and 10 mM Tris · Cl (pH 7.4). Before addition of uncoupler, mitochondria were incubated for 2 min with 2 mM ATP.

Experiments of swelling of mitochondria with BBTU were carried out as in [17], in 145 mM potassium acetate containing 5 mM Tris · Cl (pH 7.4).

The ionization constant (pK_a) of BBTU was determined spectrophotometrically as 10.3 using a Union spectrophotometer, model SM-4012.

^1H NMR spectra of BBTU and BrPh-NCS were measured with a JEOL, model PS-100, using trimethylsilane as an internal standard.

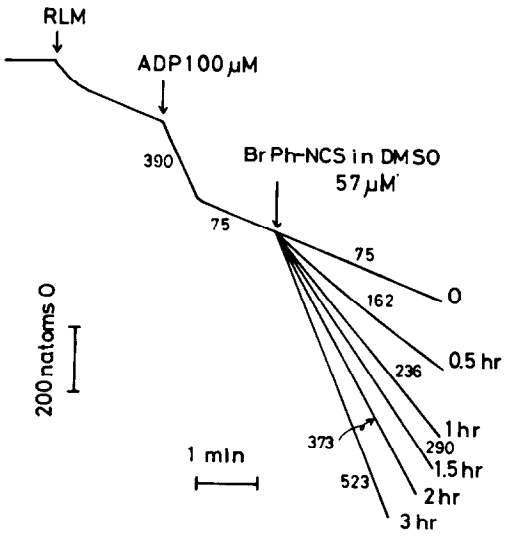

Fig.1. Effect of BrPh-NCS on the respiration of state 4 mitochondria. The stock solution of BrPh-NCS was made by dissolving BrPh-NCS in DMSO. Times (h) indicated in the figure are times after making the stock solution at 30°C. Numbers adjacent to the traces are respiratory rates in natoms 0/min. Substrate: 10 mM succinate with 3 μg rotenone. Mitochondria (RLM): 0.7 mg protein/ml.

3. Results

BrPh-NCS in DMSO was added to state 4 mitochondria to 57 μM, which is about the concentration required for maximal stimulation of respiration with succinate as substrate according to [11]. As shown in fig.1, a freshly prepared solution of BrPh-NCS had no activity on state 4 mitochondria, but a solution that stood at ~30°C for 30 min did have activity. The activity increased with time after making the solution, reaching a maximum after ~20 h. These results suggested that BrPh-NCS changed in DMSO and that the reaction product had activity on mitochondria.

To confirm this, solutions of BrPh-NCS in DMSO of various ages were subjected to thin-layer chromatography using silica gel as a stationary phase and benzene as a mobile phase. Freshly prepared BrPh-NCS solution gave one spot, but a solution that stood for ~30 min gave a second spot with a lower R_F value. With aging of the DMSO solution, the second spot became denser, while the first spot became fainter, and after 24 h, the latter had disappeared completely. Moreover, ^1H NMR spectrum of an aged solution of BrPh-NCS in DMSO showed two new peaks at δ 9.92 (NH) and δ 7.45 (aromatic protons) in addition to the original aromatic peaks of BrPh-NCS. These results show that a new compound was formed from BrPh-NCS.

The ^1H NMR spectrum, infrared spectrum and elementary analysis of the compound obtained from a solution of BrPh-NCS in DMSO that had been stood for >24 h indicated that the newly formed compound was the hydrophobic thiourea *N,N'*-bis(4-bromophenyl)thiourea (BBTU), which would be formed by the reaction of BrPh-NCS with DMSO according to the reaction scheme [18]:

$$2\text{BrPh-NCS} \xrightarrow{\text{DMSO}} \text{BrPh}-\text{NH}-\underset{\underset{\text{S}}{\|}}{\text{C}}-\text{NH}-\text{PhBr (BBTU)}$$

BBTU stimulated state 4 respiration with either glutamate plus malate, or succinate as substrate. Figure 2 shows the titration curve of state 4 mito-

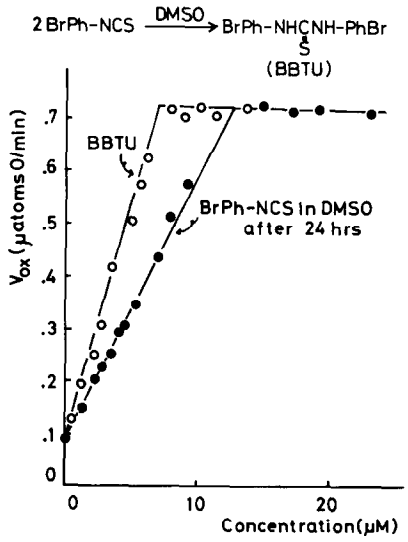

Fig.2. Titration of state 4 mitochondria with BBTU and BrPh-NCS dissolved in DMSO 24 h earlier. Experimental conditions as in fig.1.

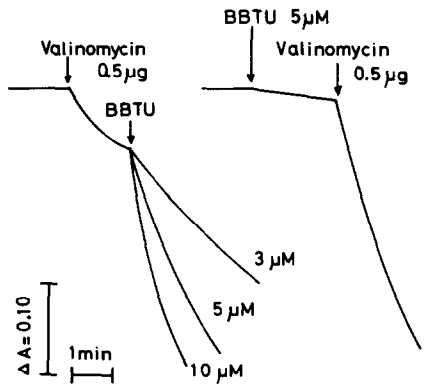

Fig.3. Acceleration of the valinomycin-induced swelling of non-respiring mitochondria by BBTU. Mitochondria (1 mg/ml) were incubated in 3.0 ml medium with 2 μg rotenone and 2 μg antimycin before addition of BBTU or valinomycin. The extent of swelling is expressed as decrease in A_{520}.

chondria with BBTU using succinate as substrate, and the effect of a solution of BrPh-NCS in DMSO prepared 24 h earlier. BBTU induces maximal release of respiration at ~7 μM and BrPh-NCS in DMSO at ~13 μM. Thus BBTU is twice as effective as BrPh-NCS in DMSO, confirming that 1 mol BBTU is formed from 2 mol BrPh-NCS.

BBTU completely released oligomycin-inhibited respiration with either glutamate plus malate, or succinate as substrate, and it activated ATPase in mitochondria at ~10 μM, as observed with commonly used weakly acidic uncouplers. These results indicate that BBTU is a potent uncoupler of oxidative phosphorylation in mitochondria.

Figure 3 shows the effect of BBTU on the passive swelling of nonrespiring mitochondria, measured as dcrease in A_{520} [17]. It can be seen that BBTU accelerated valinomycin-induced swelling and that BBTU alone did not induce swelling. This acceleration is commonly observed with weakly acidic uncouplers and is thought to result from facilitation of H^+-transport across the mitochondrial membrane by uncouplers [2–4,17].

Figure 4 shows the effect of BrPh-NCS on the respiration of state 4 mitochondria with succinate as substrate. For this experiment BrPh-NCS was dissolved in acetone, in which it was found to be very stable. Addition of 57 μM BrPh-NCS had no effect, but addition of >115 μM BrPh-NCS stimulated state 4 respiration. The effect of BrPh-NCS was time dependent after its addition: just after its addition there was no effect, but after a certain lag phase the respiratory rate gradually increases, finally reaching the maximum value. At all concentrations the maxi-

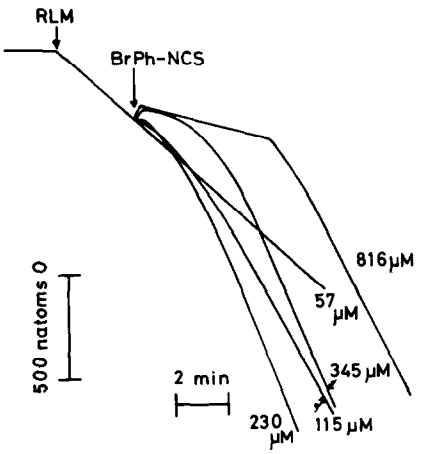

Fig.4. Effect of BrPh-NCS in acetone on state 4 mitochondria. Experimental conditions were as in fig.1.

mum value was never >3-fold the rate in state 4. The lag phase became longer as the concentration of BrPh-NCS was increased. About 50 μM BrPh-NCS, which according to [11] caused maximal release of the respiration, had no effect on mitochondria.

4. Discussion

From the findings in this study, it is concluded that BrPh-NCS changes to BBTU when dissolved in DMSO, and that BBTU has an effect on mitochondria at <50 μM. BBTU, at ~7 μM, stimulated state 4 respiration >7-fold, released oligomycin-inhibited respiration completely and activated ATPase, indicating that it is a potent uncoupler of oxidative phosphorylation in mitochondria. Since BBTU is a weak acid with pK_a 10.3, and accelerates the swelling of mitochondria induced by valinomycin, its protonophoric action is probably very important for its uncoupling activity.

As shown in fig.4, BrPh-NCS did stimulate state 4 respiration to a certain extent at $\gtrsim$100 μM. At present it is not certain whether this stimulation is actually due to uncoupling by BrPh-NCS itself. Isothiocyanates are known to be easily transformed to thioureas and dithiocarbamates by interacting with $-NH_2$ and $-SH$ groups, respectively, under mild conditions [19,20]. Even in ethanol, BrPh-NCS reacted slowly with the solvent, forming a new compound, probably the thiourethan. Thus it is possible that the limited stimulation of state 4 mitochondria by a high concentration of BrPh-NCS is due to hydrophobic, diffusible weakly acidic thiourea or dithiocarbamate formed by reaction of BrPh-NCS with non-proteinous compounds containing $-NH_2$ or $-SH$ groups, as suggested [4]. Thus the mechanism of action of hydrophobic isothiocyanates is very complex and rigorous studies are required to clarify their mechanism of action.

Acknowledgements

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