EFFECTS OF UNCOUPLER ON RAT LIVER MITOCHONDRIA ISOLATED AFTER CHLORAMPHENICOL TREATMENT

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1. Introduction

The inhibition of mitochondrial protein synthesis by chloramphenicol results in decrease of the contents of cytochromes *a*, a_3, *b* and c_1 without affecting the synthesis of cytochrome *c*, both in yeasts and in mammals [1, 2]. In addition, when the yeast *Saccharomyces cerevisiae* is grown in the presence of chloramphenicol, both the mitochondrial ATPase activity and the percentage inhibition of ATPase by oligomycin are decreased [3]. On the other hand, it has been reported that the uncoupler of oxidative phosphorylation, 5-Cl,3-*t*-butyl,2′-Cl,4′-NO_2-salicylanilide (S-13) is specific for the energy conservation site in the cytochrome oxidase region, and is bound in a one-to-one stoichiometry with cytochrome *a* [4]. This phenomenon can possibly be used to elucidate the role of mitochondrial protein synthesis in the formation of the respiratory chain.

In this communication the release of azide inhibition of state 3 respiration and the activation of the mitochondrial ATPase have been titrated with S-13. Chloramphenicol treatment in vivo did not decrease the number of binding sites of S-13 whether expressed in relation to mitochondrial protein or cytochrome *c*. However, the stoichiometry of S-13 to cytochrome *a* was drastically affected by chloramphenicol. The results serve to explain the question of the location of the synthetic apparatus for the energy transducing component at site 3.

2. Materials and methods

Five-day old rats were used as experimental animals. Chloramphenicol sodium succinate was injected intraperitoneally every six hours beginning a few hours after the birth. The total daily doses were adjusted to the elimination rate of chloramphenicol [5] according to the following schedule: 1st day, 0.62 μmoles/g body weight, 2nd day, 1.24 μmoles/g body weight, 3th–5th day, 2.48 μmoles/g body weight. Liver mitochondria were isolated in 270 mM sucrose, 1 mM EDTA, pH 7.4, as described earlier [2]. The cytochrome content of the mitochondria was determined with a dual wavelength spectrophotometer. The millimolar absorbance coefficients used were 14.0 for cytochrome *a* (605–630 nm) [6], 18.0 for cytochrome *c* (550–535 nm) [2] and 20.0 for cytochrome *b* (563–575 nm) [7]. The ATP hydrolysis was measured with a recording pH-meter [8] and the oxygen consumption was assayed with a Clark-type electrode. Succinate dehydrogenase was assayed according to Lee et al. [9]. The millimolar absorbance coefficient used for the formazan derivative was 8.0 at 500 nm.

3. Results

In mitochondria from chloramphenicol-treated animals the increase of state 4 respiration in the presence of 10 mM succinate and 2 μM rotenone was proportional to the amount of S-13 added, until the respiratory control was completely abolished. The calculated turnover number of the high-energy intermediate per molecule of uncoupler was 75 ± 10 sec^{-1}, while

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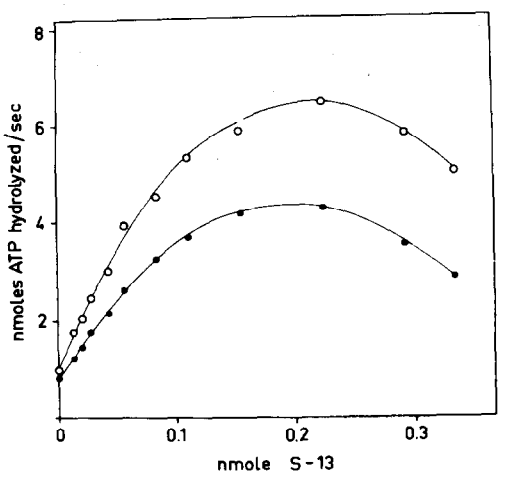

Fig. 1. Titration of the activation of mitochondrial ATPase by S-13. The assay medium was 230 mM sucrose, 2 mM ATP, 20 mM KCl, 0.1 mM EDTA (pH 7.1). The mitochondria from chloramphenicol-treated animals contained 0.20 (0.20) nmoles cytochrome *c*, 0.05 (0.21) nmoles cytochrome *a*, 0.04 (0.13) nmoles cytochrome *b* and 0.76 (0.84) mg protein (values for mitochondria from control animals in brackets). (○) control, (●) mitochondria obtained from animals treated with chloramphenicol. The initial rates of ATP hydrolysis after addition of uncoupler are shown.

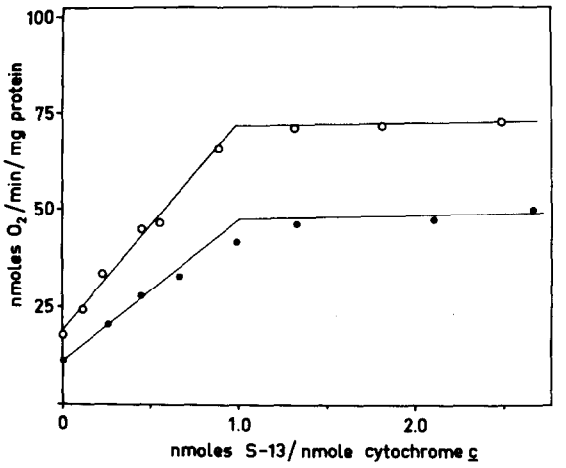

Fig. 2. Titration of S-13-induced release of azide inhibition of state 3 respiration of mitochondria. The assay conditions were: 100 mM sucrose, 25 mM tricine, 7 mM potassium phosphate, 5 mM $MgCl_2$, 30 mM KCl, 0.5 mM EDTA (pH 7.4), 10 mM succinate, 2 μM rotenone and 0.5 mM azide. (○) control, (●) mitochondria obtained after treatment with chloramphenicol. The preparations used were the same as in fig. 1.

the value in the controls was 82 ± 8 sec^{-1}; the difference is not statistically significant. The maximum rate of respiration in the presence of the uncoupler was 220 ± 15 natoms oxygen/min/mg protein in mitochondria from chloramphenicol-treated animals and 254 ± 18 natoms oxygen/min/mg protein in mitochondria from control animals. Measured by means of phenazine metholsulfate and *P*-iodonitrotetrazolium violet as electron acceptors the activity of succinate dehydrogenase in mitochondria from chloramphenicol treated animals was 166 ± 18 nmoles/min/mg protein, the corresponding activity for the controls was 160 ± 18 nmoles/min/mg protein.

The mitochondrial ATPase was activated by S-13, as shown in fig. 1. With mitochondria from control animals the slope of the line of reaction rate versus amount S-13 was independent of the amount of mitochondria added, which indicated that the unspecific binding of the uncoupler was negligible. The typical biphasic effect of the uncoupler, with inhibition commencing at higher concentrations, was observed in both kinds of mitochondria. The activation of ATPase was always sensitive to oligomycin. In fig. 1 it can be seen that in mitochondria from chloramphenicol-treated animals the maximum activity ATPase and the increase of ATPase per molecule of S-13 were 67% and 65% of the values for mitochondria from control animals, respectively. In comparison, the state 3 respiration in the presence of 10 mM succinate and 2 μM rotenone was 95 ± 5 natoms oxygen/min/mg protein after chloramphenicol treatment, while in the controls the corresponding activity was 186 ± 8 natoms oxygen/min/mg protein. The addition of ATP (2 mM) did not change the observed differences in the respiratory rates between the two groups.

An analogous situation exists when the release of azide inhibition of respiration was titrated with S-13, as shown in fig. 2. On a cytochrome basis, the end-points of the titration in the chloramphenicol-treated mitochondria (control values in brackets) were: 3.67 (0.92) S-13/cyt. *a*, 5.05 (1.62) S-13/cyt. *b* and 0.98 (0.97) S-13/cyt. *c*.

4. Discussion

In the present study the important finding of Wilson and Azzi of the stoichiometric binding of S-13 to a component of the respiratory chain have been extended to determination of the locus of synthesis of this component and to a possible discrimination of this component from the cytochromes. The titration experiments show that the number of mitochondrial binding sites of S-13 is not affected by chloramphenicol, which inhibits the synthesis of the respiratory chain cytochromes except cytochrome *c*. On the basis of the preferential inhibition of mitochondrial protein synthesis by chloramphenicol [10], it is suggested that the component with the binding site for S-13 is probably synthesized outside the mitochondria. This component, which normally appears in a stoichiometric amount with cytochrome *a*, loses this stoichiometry when the animals are subjected to chloramphenicol treatment, but still holds the stoichiometry with cytochrome *c*. In view of the correlation of this component with azide inhibition of the electron and energy transport at site 3 [4, 11], it is plausible to assume that this uncoupler-binding component is specific to site 3.

If the possibility of preferential inhibition by chloramphenicol of the synthesis of the heme groups of cytochrome aa_3 can be excluded, it is also evident that the apoprotein portion of cytochrome oxidase is not the component providing the site for the uncoupler. On the other hand, the presence of the apoprotein portion of cytochrome aa_3 after chloramphenicol treatment would indicate that mitochondrial protein synthesis of heme components of cytochrome oxidase. According to the results obtained with petite mutants of yeast [12–14] the latter alternative is more feasible.

In mitochondria obtained after chloramphenicol treatment, the state 3 respiration in the presence of succinate and rotenone was lower than in the controls. The percent decrease (49%) of state 3 respiration by chloramphenicol treatment is somewhat more than the concomitant decrease of the uncoupler-stimulated ATPase activity (33%) and much more than the decrease of the uncoupled respiratory rate (13%) after chloramphenicol treatment as compared to the controls. This suggests that, besides the concentration of cytochromes, the amount of the energy-transducing component is the limiting factor in the state 3 respiration in mitochondria of chloramphenicol-treated animals.

According to Schatz [3], the decrease of ATPase activity during chloramphenicol treatment in yeast is probably not due to defective synthesis of the soluble ATPase (F_1), but to a deficiency in the membrane component of mitochondrial ATPase (CF_0) giving rise to oligomycin sensitivity. The possible identity of F_1 as a binding site for S-13 needs further clarification. This is important because dinitrophenol has a small effect on the ATPase of F_1 isolated from mammalian mitochondria as compared to F_1 isolated from yeast [15].

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