2,4 BIS (*p*-CHLOROANILINO)-PYRIMIDINE, AN UNCOUPLER OF OXIDATIVE PHOSPHORYLATION

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1. Introduction

Studies on the synthesis and antibacterial properties of substituted pyrimidines like 2,4-bis-(arylamino)-6-hydroxy pyrimidines were reported by Roy et al. [1,2]. Ghosh [3] reported the synthesis of several 2,4-bis-(arylamino) pyrimidines and also the antimicrobial properties of these compounds. 2,4-bis-(*p*-chloroanilino)-pyrimidine, being the most potent inhibitor of growth of yeasts and bacteria, was chosen for studies on the mechanism of its action. The effect of this compound on the respiratory activity of yeast cells and rat liver mitochondria is reported in this paper. This compound is found to have a partial uncoupling effect on oxidative phosphorylation.

2. Methods and materials

Brewers yeast, *S. carlsbergensis*, Hillman Hospital strain 4228, was obtained from ATCC and grown in liquid medium as described by Ghosh et al. [4]. The cells were harvested from the log phase (14 hr growth), washed twice with distilled water and then suspended with twice the volume of 0.1 M KH_2PO_4. The cell suspension was aerated for 3 hr and then washed with distilled water and resuspended in 0.1 M KH_2PO_4 as before and aeration continued till use. 0.1 M KH_2PO_4 was used as the medium for studies on yeast respiration.

Rat liver mitochondria was prepared by the method of Chance and Hagihara [5], using 10^{-4} M EDTA. The final washings were done in the absence of EDTA. The mitochondria were suspended in 0.225 M mannitol and 0.075 M sucrose medium. Reaction medium for the respiration studies was 0.225 M mannitol, 0.075 M sucrose, 10 mM Tris-phosphate and 10 mM Tris-chloride and the pH was adjusted to pH 7.2.

The oxygen uptake by yeasts and mitochondria was measured polarographically with a Clark oxygen electrode connected to a Servo-Riter pen recorder (Texas Instrument Inc.), the polarising voltage being −0.6 V.

Oligomycin and antimycin A (Sigma Chemical Company) and also 2,4-bis-(*p*-chloroanilino)-pyrimidine (BCAP) and *p*-trifluoromethoxy-carboxycyanide phenylhydrazone (FCCP) were used in ethanolic solutions.

3. Results

The effect of BCAP on the respiration of yeast using ethanol as substrate is given in fig. 1. The endogenous respiratory rate and that due to ethanol were 13 and 25 μM O_2 per min respectively. 40 μM

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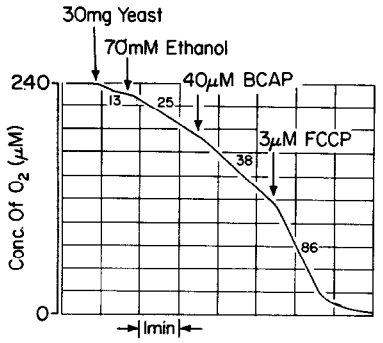

Fig. 1. Oxygen electrode trace of ethanol oxidation by *S. carlsbergensis* (30 mg wet wt) suspended in 3,5 ml of 0.1 M KH_2PO_4, pH 4.6. The respiratory rates in $\mu M\ O_2$/min after additions of yeast, ethanol, BCAP and FCCP are given in the figure.

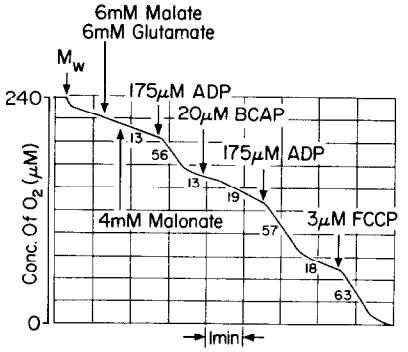

Fig. 2. Oxygen electrode trace of glutamate + malate oxidation by rat liver mitochondria (4 mg protein) suspended in 3.5 ml of 0.3 M mannitol-sucrose medium containing 10 mM Tris-phosphate and 10 mM Tris-chloride buffered to pH 7.2. Numbers on the trace indicate rates of oxygen uptake in $\mu M\ O_2$/min at different states.

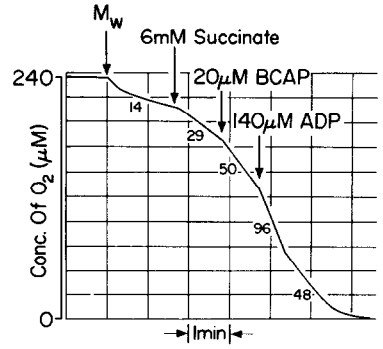

Fig. 3. Oxygen electrode trace of succinate oxidation by rat liver mitochondria (5.5 mg protein) suspended in 3.5 ml medium as stated in fig. 2. Numbers in the figure indicate the rates of oxygen uptake in $\mu M\ O_2$/min due to additions of succinate, BCAP, ADP and also the rate after ADP exhaustion.

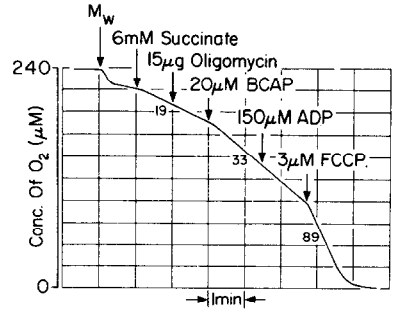

Fig. 4. Oxygen electrode trace showing the effects of oligomycin, BCAP, ADP and FCCP on succinate oxidation by rat liver mitochondria (5 mg protein) suspended in 3.5 ml medium as stated in fig. 2. The numbers in the figure indicate the rates of oxygen uptake in $\mu M\ O_2$/min due to succinate, BCAP and FCCP. The state-4 rate due to succinate was unaffected by oligomycin and the BCAP-stimulated rate was unaltered by ADP.

BCAP increased the rate to 38 and 3 μM FCCP stimulated the rate further to 86 $\mu M\ O_2$ per min. The ratio of FCCP stimulated rate to BCAP stimulated rate was 2.5. In separate experiments it was observed that the BCAP stimulated oxygen uptake was inhibited by both antimycin A (5 μg/ml) and sodium azide (20 μM).

The effect of BCAP on malate and glutamate oxidation by rat liver mitochondria is given in fig. 2. Prior to the addition of BCAP, the respiratory control ratio was 4.3 and ADP:O ratio was 2.6. Addition of 20 μM BCAP stimulated the rate from 13 to 19 $\mu M\ O_2$ per min. 175 μM ADP showed the usual state 4-3-4 transition but a lower ADP:O ratio of 1.7. The state-4 rate was the same as the BCAP-stimulated rate. FCCP uncoupled the mitochondria completely and the oxygen uptake rate increased to 63 $\mu M\ O_2$ per min, a little higher than the ADP-stimulated rate. The ratio of ADP-stimulated rate to BCAP-stimulated

rate varied between 2.9 to 3.0 in different experiments.

The effect of BCAP on succinate oxidation by rat liver mitochondria is given in fig. 3. 20 μM BCAP increased the state-4 rate from 29 to 50 μM O_2 per min. The rate after ADP (140 μM) addition and exhaustion were 96 and 48 μM O_2 per min respectively and the ADP:O ratio decreased to 1.0. In parallel experiments, in the absence of BCAP, the ADP:O ratios were 1.7–1.8. The ratio of ADP-stimulated rate to BCAP-stimulated rate varied between 1.7 to 1.9 in different experiments.

The effect of BCAP on oligomycin-treated mitochondria is given in fig. 4. Addition of oligomycin (5 μg/ml) did not affect the state-4 rate due to succinate but 20 μM BCAP increased the rate from 19 to 33 μM O_2 per min. ADP did not affect this rate but 3 μM FCCP enhanced the rate to 89 μM O_2 per min, which is 2.7 times the rate due to BCAP. This ratio, however, was higher than that due to ADP- and BCAP-stimulated rates.

In separate experiments it was observed that the respiration of rat liver mitochondria stimulated by BCAP was inhibited by both antimycin A (5 μg/ml) and sodium azide (15 μM).

4. Discussion

Results clearly showed that BCAP stimulated the rate of oxygen uptake by *S. carlsbergensis* using ethanol as the substrate. Further stimulation of the rate was observed with FCCP. No conclusions could be reached regarding the mechanism of BCAP effect as the enhanced rate of electron flow through the respiratory chain may either be due to the activation of the dehydrogenases and other electron transport enzymes or to the uncoupling of respiration.

Studies on the effect of BCAP on rat liver mitochondria clearly showed that the enhancement of oxygen uptake rate was associated with a drop in ADP:O ratio by nearly 1. The drop in ADP:O ratio due to BCAP and still observable state-4-3-4 transition effected by ADP in both succinate- and NAD-linked electron transport pathways, are believed to be due to uncoupling of one site of phosphorylation by BCAP and that site is located in between cytochromes b and a.

In oligomycin-treated rat liver mitochondria BCAP stimulated the respiratory rate but ADP had no effect on the rate, whereas FCCP uncoupled the oligomycin effect. This further established that BCAP uncoupled one site leaving the other untouched where ADP phosphorylation was blocked by oligomycin.

The inhibition of BCAP-stimulated respiration by antimycin A and sodium azide clearly indicates that under this state the electrons flow through the usual cytochrome chain and not through any bypass of an energy conservation site.

The ratio of ADP-stimulated rate to BCAP-stimulated rate was found to be 1.7–1.9 for succinate oxidation and 2.8–3.0 for malate + glutamate oxidation. Simple calculations suggest that the site of energy conservation, 2 or 3, which is not uncoupled by BCAP has the maximum control on coupled electron flow through the respiratory chain. The relative control efficiencies are calculated to be 1.0 to 1.1 for site 1, 1.8 to 1.9 for the site unaffected by BCAP and 1.0 for the site uncoupled by BCAP.

It is quite interesting to note that a mild uncoupling agent like BACP which keeps the ATP synthesis in somewhat lower level, can effectively inhibit the growth of organisms. Studies on its effect on the glycolysis and protein metabolism and also on the location of the site of action of BCAP in the respiratory chain are underway.

Acknowledgements

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