INHIBITION OF SUCCINATE OXIDATION IN MITOCHONDRIA BY BROMOTHYMOL BLUE

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1. Introduction

Chance and co-workers [1–6] have shown that bromothymol blue (BTB) can be used to indicate the pH on the inside of the cristae membranes of intact respiring mitochondria. It has also been observed that BTB is bound to the membranes of submitochondrial particles, of bacterial chromatophores and of chloroplast grana, and it has been deduced that it indicates the inside pH in these preparations [7,8]. The BTB technique for the measurement of internal pH has been generally accepted, however Mitchell [9] has shown that it is incompetent to measure the pH of the inner phase of mitochondria in state 4.

This paper describes the inhibition by BTB of succinate oxidation in intact rat liver mitochondria in state 3, in the uncoupled state and in the ion-pumping state and the lack of any effect of this dye on succinate oxidation in sonic particles. The results obtained suggest that the inhibition is caused by a direct action of BTB on succinate transport into the mitochondrion, and that the use of BTB for the measurement of internal pH may be complicated by the action of BTB on succinate transport, if this anion is used as the substrate.

2. Materials and methods

Succinic acid, rotenone and gramicidin were obtained from the Sigma Chemical Co.; 2,4-dinitrophenol from AG Fluka; ADP from Calbiochem; and bromothymol blue from Merck AG.

Rat liver mitochondria were prepared as described previously [10]. Sonic particles were prepared according to Kielley and Bronk [11]. Protein was estimated by the biuret method [12]. Respiration rates were measured with a Clark oxygen electrode. The reaction mixture (final vol 3.5 ml) contained: 15 mM KCl, 50 mM Tris-HCl pH 7.3, 5 mM $MgSO_4$, 1 mM potassium phosphate, 4 μg rotenone, and 6 mg mitochondrial protein. Respiration was stimulated by the addition of 1 mM ADP (state 3) or 0.05 mM 2,4-dinitrophenol (uncoupled state). Oxygen uptake in the ion-pumping state was measured in a reaction mixture containing 250 mM sucrose, 20 mM Tris-HCl pH 7.3 , 10 mM KCl, 5 mM Tris-acetate, 0.5 μg gramicidin/ml and 4 μg rotenone. Succinate was used as the potassium salt at concentrations indicated on the figures, BTB was dissolved in ethanol and 10 μl added to the medium.

3. Results and discussion

The addition of BTB to a suspension of liver mitochondria oxidizing succinate (1 mM), in the presence of ADP and phosphate, or 2,4-dinitrophenol caused immediate inhibition of respiration (figs. 1a,b). Addition of 3 mM succinate released this inhibition. The inhibition of uncoupled succinate oxidation by BTB and the decreased inhibition when the succinate concentration was increased suggest that BTB acts on the transport of succinate into the mitochondrion. According to Quagliariello and Palmieri [13], measurement of the rate of succinate oxidation in the presence of rotenone is a simple way of studying possible interference of NAD-linked substrates with the entry or accumulation of succinate within the mitochondria. Using this method we observed that oxygen uptake by rat liver mitochondria in the ion-pumping state, with 1 mM succinate (+rotenone) in the incubation medium, was inhibited by the addition of 30 μM BTB. This inhibition was re-

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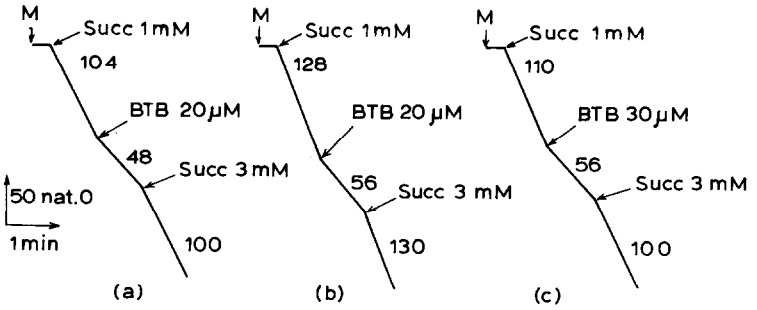

Fig. 1. Effect of bromothymol blue on succinate oxidation in intact rat liver mitochondria in: (a) active state; (b) uncoupled state; (c) ion-pumping state. Experimental conditions as indicated under Materials and methods. M, mitochondria; Succ, succinate; BTB, bromothymol blue. Oxygen consumption expressed as natoms O/min/mg protein.

leased by the addition of 3mM succinate (fig. 1c). These observations suggested that the action of BTB was on the entry of succinate into the mitochondrion, however they do not exclude a direct action of BTB on succinate dehydrogenase. As is shown in fig. 2a, BTB even at relatively high concentration was without effect on succinate (+rotenone) oxidation in sonic particles. These results suggest that BTB at this concentration does not act directly on succinate dehydrogenase. The lack of any effect of BTB on succinate oxidation in sonic particles also confirms our suggestion that the dye acts on the transport of succinate into the mitochondrion, assuming that in submitochondrial particles the concentration of succinate at the site of succinate dehydrogenase is always the same as in the incubation medium. According to Quagliariello and Palmieri [13] the influence of succinate uptake on the rate of respiration can only be observed in the presence of low concentrations of substrate. If one adds a high concentration of succinate to the incubation medium, the intramitochondrial concentration will always be higher than the minimal required for a maximal rate of respiration, thus accumulation within the mitochondria cannot be observed by measuring the rate of respiration [13]. The above data might explain why BTB was without effect on mitochondrial respiration with 5 mM succinate (+rotenone) (fig. 2b). It is known that entry of succinate into the mitochondrion takes place via the dicarboxylic acid transporter [14]. This dicarboxylic acid transporter may be inhibited by butylmalonate [15], tannic acid [16] and bathophenanthroline [17]. The question arises what is the mechanism of BTB action on succinate transport into the mitochondrion like? According to Quagliariello and Palmieri [13],

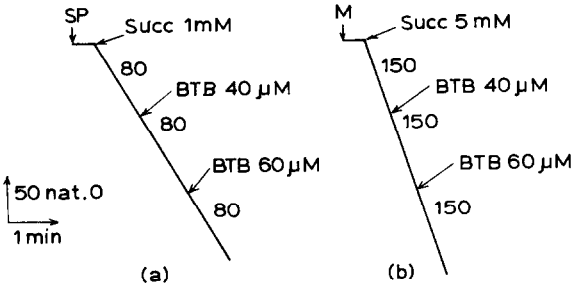

Fig. 2. Succinate oxidation by (a) sonic particles; (b) intact mitochondria in the presence of bromothymol blue. Incubation medium was 15 mM KCl, 50 mM Tris-HCl pH 7.3 5 mM $MgSO_4$, 1 mM potassium phosphate, 4 μg rotenone, plus in (a) 2 mg sonic particles protein (SP) and in (b) 6 mg mitochondrial protein (M) and 0.05 mM 2,4-dinitrophenol (DNP).Succinate and BTB final concentrations as indicated on the figure. Oxygen consumption expressed as natoms O/min/mg protein.

2,4-dinitrophenol at high concentration causes a decrease of the intramitochondrial concentration of succinate and consequently an inhibition of mitochondrial respiration, as a result of the inhibition of active ion transport. The experiments presented by Papa et al. [18] have shown that uncouplers can act in two ways on the transport of anionic substrates. Firstly, they promote the efflux of P_i from mitochondria, but not directly that of a dicarboxylate or tricarboxylate. Secondly, uncouplers inhibit the transport of anionic substrates by an interaction with the translocators. It has been shown [19] that BTB binds to the inner mitochondrial membrane. As was shown previously, carrier systems for the transport of Krebs cycle intermediates are located in this membrane [14–18]. We assume that BTB, reacting with the dicarboxylic acid carrier system,

Table 1
Effect of varying concentrations of bromothymol blue (BTB) on succinate oxidation in the uncoupled state and the ion-pumping state.

BTB concentration (μM)	Inhibition	
	Uncoupled state (%)	Ion-pumping state (%)
5	10	
10	25	12
20	56	30
30	75	50
40	95	77

Experimental conditions as presented under Materials and methods. Succinate: 1 mM.

inhibits succinate transport into the mitochondrion. The inhibitory effect of BTB on succinate transport is not specific because we observed also that this compound inhibits transport of other anions into the mitochondrion. The sensitivity of 1 mM succinate (+ rotenone) oxidation to different concentrations of BTB in the uncoupled state was compared to that in the ion-pumping state. As is shown in table 1, 5 μM BTB produced a slight inhibition of succinate oxidation in the uncoupled state. This inhibition increased proportionally when the concentration of BTB was increased. In the ion-pumping state at 5μM BTB we did not observe inhibition of 1 mM succinate oxidation. 10 μM BTB caused negligible inhibition in this state but the degree of inhibition increased with increasing BTB concentrations. Azzone et al. [20] observed that the binding of BTB to rat liver mitochondria is higher in KCl than in sucrose medium. This might explain why the degree of inhibition of succinate (+rotenone) oxidation by the same concentration of BTB is smaller in the ion-pumping state (250 mM sucrose) than in the uncoupled state (without sucrose). Other results (not shown here) indicate that the percentage of inhibition of succinate oxidation caused by the same concentration of BTB in mitochondria uncoupled either by 0.05 mM 2,4-dinitrophenol or 0.5 μg/ml gramicidin in identical media was very similar. Although the BTB was usually used at a concentration of 3.3 μM to measure the pH of the inner phase, it was, however, also applied at higher concentrations. Chance [21], to measure extramitochondrial pH changes caused by Ca^{2+} uptake in the presence of phosphate in guinea pig kidney mitochondria, used BTB at a concentration of about 10 nmoles/mg protein. Azzone et al. [20] used 10 μM BTB and 200 μM succinate in measuring the BTB response induced by succinate in KCl medium. In our experiments BTB at a concentration 10 μM caused immediate inhibition of 0.2 mM succinate oxidation (not shown here). It must therefore be expected that the BTB technique for measurement of internal pH of intact respiring mitochondria with succinate as substrate may be complicated by the action of the dye on succinate transport into the mitochondrion, if succinate is used at lower and BTB at higher concentrations.

Acknowledgements

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