AZIDE INHIBITION OF MITOCHONDRIAL ATPase

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In intact mitochondrial systems azide has been reported as an inhibitor of cytochrome oxidase (Keilin, 1936; Yonetani and Ray, 1965), an uncoupler of oxidative phosphorylation (Loomis and Lipmann, 1949; Judah, 1951; Slater, 1955), and an inhibitor of ATPase (Robertson and Boyer, 1955). Judah (1951) and Robertson and Boyer (1955) reported that azide inhibition of the exchange reactions was associated with energy transfer. Palmieri and Klingenberg (1967) have suggested that azide acts on cytochrome a only.

Results presented here show that the amount of azide required to inhibit succinate oxidation in tightly coupled mitochondria respiring in state 3 is also sufficient to inhibit purified ATPase. Furthermore, azide inhibition of ATPase activity is enhanced in the presence of ADP to an extent unaccountable on the basis of mass action alone. These findings indicate that azide has an activity on energy transfer which is distinct from azide binding with cytochrome a.

Methods

Beef heart mitochondria were prepared according to Haas and Elliot (1963). Pat liver mitochondria were prepared as described previously (Ziegler et al., 1965). Mitochondrial ATPase was prepared essentially as outlined by Penefsky et al. (1960) with the following variations. The mitochondria were suspended (0.4 g/ml) in a sonication medium containing 0.4 M $(NH_4)_2SO_4$, 0.25 M sucrose, 0.01 M Tris, and 0.001 M EDTA, pH 7.4. Sonication was carried out for 10 seconds at one minute intervals for a total of 50 seconds (Branson Sonic Oscillator). During this procedure the sonication vessel was immersed in an ethanol / dry ice bath at - 10°, maintaining the mitochondrial suspension at 0° (Gregg, 1963). The sonicated suspension was centrifuged at 105,000 x g for 35 minutes and the pale yellow supernatant was applied to a Sephadex G-100 column (1.5 x 90 cm) previously equilibrated in a solution of 0.1 M sodium acetate, 0.1 M sodium bicarbonate, and 1 mM ATP. Elution was carried out with a 0.1 M sucrose, 5 mM Tris, and 4 mM ATP solution. Protein concentration of the eluate was followed at 260 mμ, using the appropriate extinction coefficient. The enzyme appeared in the first protein band and was contained in six to seven ml. The enzyme was precipitated by addition of two volumes of saturated $(NH_4)_2SO_4$, followed by centrifugation at 105,000 xg. Ammonium sulfate was decanted and the enzyme was suspended in 0.25 M sucrose, 0.05 M Tris, and 0.1 M $MgCl_2$, pH 7.4 when used immediately. Otherwise it was stored at room temperature in the sonication medium. Sodium azide was obtained from Eastman and used without further purification. ATPase activity was determined by release of inorganic

phosphate. Phosphate was measured by the method of Chen et al. (1956), and protein was measured according to Lowry (1951).

Results and Discussion

The results depicted in Fig. 1 demonstrate the inhibition of purified ATPase by azide. Some inhibition of the ATPase from beef heart mitochondria was observed at 10^{-6} M azide, while ATPase from rat liver mitochondria was not affected by azide at this concentration. However, ATPase from both rat liver and beef heart mitochondria were strongly

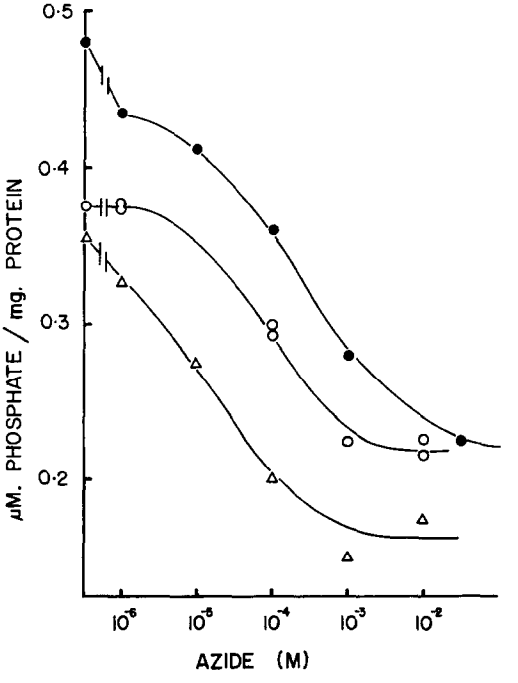

Fig. 1

Effect of sodium azide on the hydrolysis of ATP by purified ATPase from beef heart and rat liver mitochondria. Hydrolysis of ATP in the presence of azide and ADP is also shown. Incubation medium, .25 M sucrose, .05 M Tris, .1 mM $MgCl_2$, 1 mM ATP, pH 7.4, ADP when used was 50 µM. Incubation time, 15 minutes, temperature, 22°. (●----●) Beef heart ATPase. (0----0) Rat liver ATPase. (Δ ----Δ) Rat liver ATPase in the presence of ADP.

inhibited by azide at concentrations above 10^{-5} M, with maximal inhibition being given by about 10 mM azide. Half maximal inhibition was given by about 100 μM azide in both rat liver and beef heart ATPase.

Wilson and Chance (1967) observed that mitochondria respiring in the presence of substrate and ADP (state 3) are more sensitive to azide than mitochondria respiring in the presence of substrate alone (state 4). Therefore, the sensitivity of ATPase to azide was tested in the presence of 50 μM ADP. In the presence of ADP, rat liver mitochondrial ATPase was more sensitive to azide (10^{-6} M) as seen in Fig. 1.

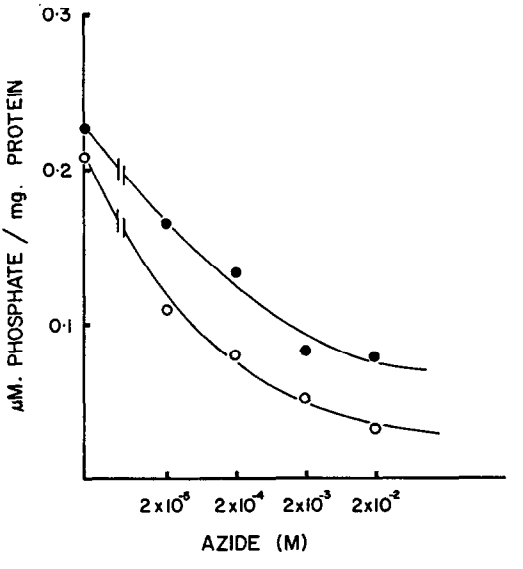

Fig. 2

Effect of sodium azide on the hydrolysis of ATP in rat liver mitochondria uncoupled with 100 μM 2,4-dinitrophenol in the absence of ADP (●----●) and with 50 μM ADP in the incubation medium (0----0). Conditions of incubation were the same as in Fig. 1.

Maximal inhibition was given between 1 to 10 mM azide and the azide concentration needed for half maximal inhibition in the presence of ADP was 30 μM. Azide inhibition of DNP-stimulated ATPase in intact mitochondria was also increased in the presence of ADP (Fig. 2).

The results of this study support the conclusion that azide is an inhibitor of energy transfer. This effect is distinct from the binding of azide to cytochrome a reported by Wilson and Chance (1967). The fact that azide at low concentrations did not stimulate isolated ATPase confirms the earlier conclusion (Penefsky et al., 1960) that this enzyme is the DNP-stimulated ATPase of intact mitochondria rather than Mg^{2+}-stimulated ATPase. A dual activity of azide on energy transfer as suggested by Bogucka & Wojtczak (1966) is compatible with the present results, since the enzyme responsible for Mg^{2+}-stimulated ATPase may be tightly bound to the mitochondrial membrane.

Negative control of respiration by ADP has been observed under conditions of inhibited phosphorylation (Ziegler et al., 1965, Vázquez-Colón et al., 1966, Wilson and Chance, 1967). It has been suggested that ADP acts to make the sensitive site on the phosphorylation enzyme(s) in intact mitochondria more accessable to the inhibitor (Vázquez-Colón et al., 1966). It is possible that ADP may facilitate the entry of azide to a sensitive site on isolated ATPase as well.

The present report and other available data lead to the following summary of azide action. First, azide has a direct effect of cytochrome oxidase; second, azide has a specific inhibitory effect on the terminal phosphorylating enzyme system; third, as concluded by Palmieri and Klingenberg

(1967), azide probably has secondary effects due to its accumulation as a permeant anion. These secondary effects may include uncoupling activity and stinulation of Mg^{2+}-activated ATPase in intact mitochondria. Further work is in progress to characterize the inhibition of ATPase by azide and to establish its relationship with the other sites of action of this inhibitor.

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