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INHIBITION OF SOLUBLE YEAST MITOCHONDRIAL ATPase BY ETHIDIUM-BROMIDE

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SUMMARY

Ethidium-bromide inhibits purified yeast F_1. Its inhibition yields non competitive kinetics with ATPMg and ADPMg but is competitive with 2,4 dinitrophenol. In submitochondrial particles, ethidium-bromide is competitive with para-hydroxymercuribenzoate. These data suggest a target for ethidium-bromide located at a site of F_1 not involved in the hydrolysis of ATPMg. This non catalytic site also reacts with 2,4 dinitrophenol and with para-hydroxymercuribenzoate.

Ethidium-bromide is actively concentrated by *Saccharomyces cerevisiae* cells (1) and induces up to 100 % of the mitochondrially-inherited, respiratory-deficient, rho-minus mutation in the absence of cellular multiplication (2). Several types of experimental evidences indicate the existence of an interaction between ethidium-bromide and the mitochondrial ATPase. The *in vitro* induction by ethidium-bromide of the degradation of mitochondrial DNA depends on the synthesis of mitochondrial ATP (3, 4). A UV-sensitive diazido derivative of ethidium-bromide has been shown to bind a lipophilic site tentatively identified as the lowest molecular weight component of the oligomycin-sensitive, membrane-bound mitochondrial ATPase of *S. cerevisiae* (5). In isolated mammalian mitochondria, ethidium-bromide has been shown to bind strongly to energized membranes (6 to 8), to be actively accumulated inside mitochondria (9) and to uncouple or inhibit oxidative phosphorylation (10 to 12). A recent report indicated that ethidium-bromide inhibits slightly (25%) the mitochondrial ATPase activity in rat liver submitochondrial particles but the soluble ATPase (F_1) is barely inhibited (9).

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The above mentioned data suggest a target for ethidium-bromide on the membrane part (Fo) of the ATPase rather that on the catalytic part (F_1). However, in this communication we describe a clear inhibition of the soluble purified F_1 mitochondrial ATPase by ethidium-bromide in *S. cerevisiae*, and based on kinetic evidence, propose a target for this inhibitor located on a non-catalytic site of F_1.

MATERIALS AND METHODS

Saccharomyces cerevisiae strain *N123(a his_1)* was provided by Dr. E. Moustacchi, Fondation Curie, Orsay, France. Growth conditions in glycerol, determination of protein concentrations and preparation of sonicated submitochondrial particles exhibiting ATPase activity of about 5 µmol x min^{-1} x mg^{-1} were previously reported (13). F_1 ATPase of specific activity of 60 to 120 µmol x min^{-1} x mg^{-1} was extracted by chloroform from *N123* submitochondrial particles and further purified by anion-exchange and molecular sieve chromatography as described in Goffeau *et al.* (14). Unless otherwise indicated, the 1 ml reaction mixture of the ATPase assay incubated at 30°C for 8 min contained : 20 mM Tris-HCl pH 9.0, 3 mM $MgCl_2$, 3 mM ATP, 2 mM phosphoenolpyruvate, 10 units of pyruvate kinase (Boehringer) and 16 mM $(NH_4)_2SO_4$.

RESULTS

The double reciprocal plot obtained for 1/ATPMg in the presence of several concentrations of ethidium-bromide exhibits classic non-competitive kinetics of ethidium-bromide with respect to ATPMg, indicating no interference with the binding of ATPMg. The concentration of ethidium-bromide required for 50 % inhibition of purified yeast F_1 is 220 µM ethidium-bromide (Fig. 1).

Dinitrophenol was previously reported to activate beef-heart (15) and yeast (16) F_1. Such stimulation is not observed under our conditions for purified F_1 in absence of ethidium-bromide. However, the double reciprocal plot of Fig. 2 shows a strong interaction between dinitrophenol and ethidium-bromide. Even though no strict linearity is obtained in this plot, the intersection with the abcissa is considerably modified, whereas only minor modifications of the intersection with the ordinate (V_{max}) are observed. These data indicate that the presence of dinitrophenol markedly decreases the inhibition by ethidium-bromide in a virtual competitive fashion.

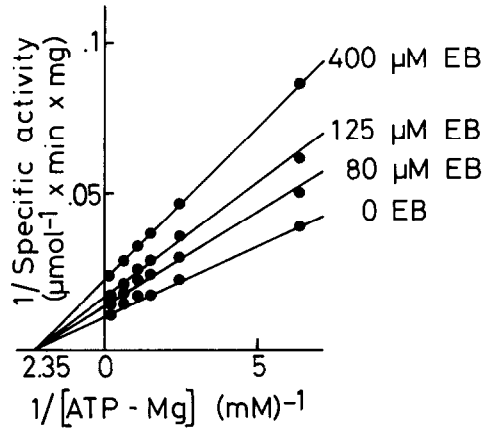

Figure 1. Double reciprocal plots of yeast F_1 specific activity versus ATPmg at different concentrations of ethidium-bromide (EB).

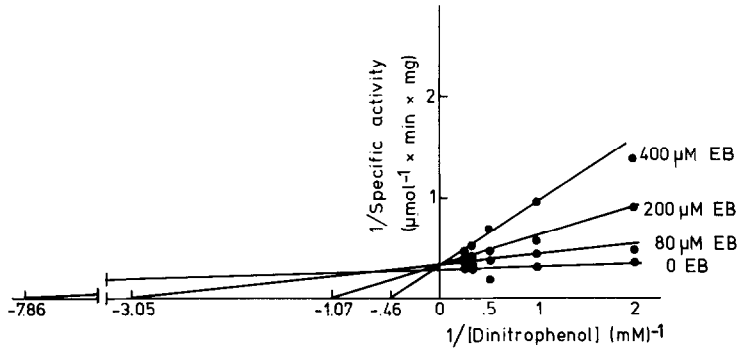

Figure 2. Double reciprocal plots of yeast F_1 specific activity versus 2,4 dinitrophenol at different concentrations of ethidium-bromide. The assay conditions were as described in Materials and Methods except that no $(NH_4)_2SO_4$ was added and that 5 μl of glycerol were introduced in the reaction mixture.

Interactions between two inhibitors are conveniently studied in Dixon plots where the reciprocal of activities measured at several fixed concentrations of one inhibitor are ploted versus increasing concentrations of another inhibitor. A family of parallel lines indicates competition between the two inhibitors, whereas lines converging on the abcissa indicate non-competitivity (17).

The Dixon plots of ADP inhibition of ATPase activity of purified F_1 in the presence of 80 μM and 125 μM ethidium-bromide (Fig. 3) indicate non-competitive kinetics of ADP and ethidium-bromide.

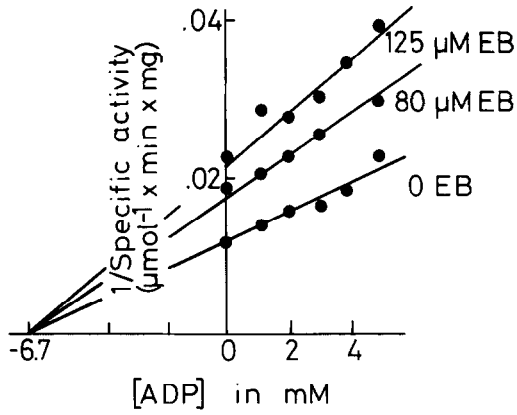

Figure 3. Dixon plots of yeast F_1 specific activity in the presence of ethidium-bromide and ADP. The assay conditions were as described in Materials and Methods except that no phosphoenolpyruvate, no pyruvate kinase and no $(NH_4)_2SO_4$ were added.

It has been shown that beef heart (15, 18) and rat liver F_1 (19) are not appreciably sensitive to sulfhydryl reagents when tested in the absence of bicarbonate anions (19). However the membrane-bound beef heart mitochondrial ATPase is very sensitive to mercurials (20). In this respect, the yeast ATPase behaves similarly to the mammalian enzyme. Moreover, in yeast submitochondrial particles the inhibition of ATPase activity by para-hydroxymercuribenzoate interferes strongly with that of ethidium-bromide as shown by the Dixon plot of Fig. 4 which indicates competitive kinetics for the two inhibitors.

DISCUSSION

Our data demonstrate that ethidium-bromide acts at the level of the F_1 part of the yeast mitochondrial ATPase. This, of course, is not in contradiction with the observation that this drug could also bind to a membrane component of the yeast mitochondrial ATPase (5).

Our data indicate that ethidium-bromide does not act at the active site of F_1, since it yields non-competitive inhibition kinetics with ATPMg as well as with ADPMg. Ethidium-bromide competes with para-hydroxymercuribenzoate and 2,4 dinitrophenol for their inhibitory site. A large body of

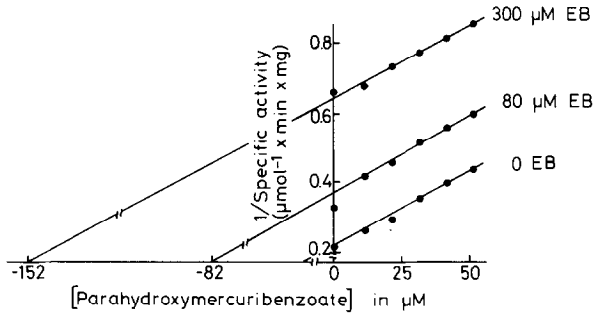

Figure 4. Dixon plots of ATPase activity of yeast submitochondrial particles in the presence of ethidium-bromide and para-hydroxymercuribenzoate.

evidences indicates that in addition to the catalytic site at least one other non-catalytic binding site for ATPMg exists in the mitochondrial ATPase (see reviews 21, 22, 23). In purified yeast F_1, recent kinetic steady-state data indicate the existence of a regulatory site for which anions compete with ATPMg (24, 25). It has been shown that the activation by 2,4 dinitrophenol of the beef heart F_1 is inhibited by para-chloromercuribenzoate (15). Activation by the bicarbonate anion of the rat liver F_1 is also inhibited by mercurials (19). These observations are consistent with the suggestion of a common site of action for ethidium-bromide, for activating anions such as 2,4 dinitrophenol, and for inhibitory mercurials such as para-hydroxymercuribenzoate. This site appears distinct from the catalytic site involved in ATPMg hydrolysis and its inhibition by ADPMg. This interpretation is illustrated in Fig. 5. If this interpretation is correct, the fluorescence properties of ethidium-bromide could be used for the identification of the non-catalytic site in the different subunits of F_1.

What is the possible physiological significance of our data ? An ATP-dependence of at least one step of the rho-minus induction process by ethidium-bromide is suggested by a body of evidences where rho-minus induction by low concentrations of ethidium-bromide was decreased by an inhibition of respiration using antimycin A (22, 27), anaerobiosis (28, 29), glucose

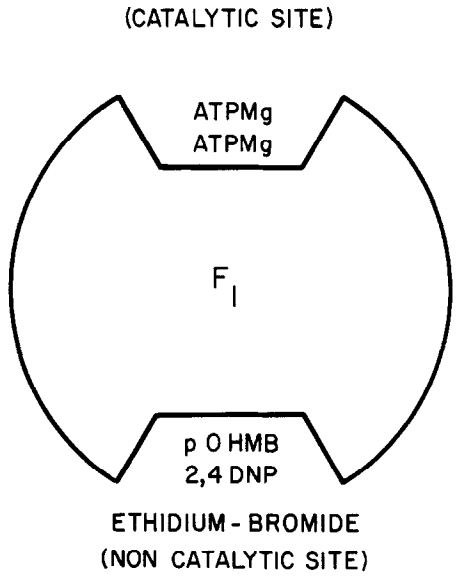

Figure 5. Schematic model of the sites of yeast F_1 involved in the inhibition by ethidium-bromide and the other effectors studied in this paper. pOHMB is para-hydroxymercuribenzoate and 2,4 DNP is 2,4 dinitrophenol).

repression (27, 30) or by treatment with oligomycin (29) or with uncouplers (27) - all of which serve to lower the intracellular and intramitochondrial concentration of ATP. It as also been observed that under appropriate conditions, ethidium-bromide at high concentrations can reverse an unstable "premutational" state, induced itself by lower concentrations of ethidium-bromide (31 to 33). This experimental observation is difficult to rationalize with a straighforward mechanism of rho-minus induction by ethidium-bromide *via* binding of the mutagen to mitochondrial DNA only. However, the results presented here demonstrate that concentrations of the order of 0.1 mM to 1 mM ethidium-bromide inhibit the mitochondrial ATPase *in vitro*. It has been estimated that intact yeast cells can accumulate up to 20 to 30 mM ethidium-bromide (1) and that intact mammalian mitochondria can accumulate up to 80 mM internal ethidium-bromide (9). We therefore suggest that the decrease in rho-minus induction at high ethidium-bromide concentrations might result from the decrease in mitochondrial ATP due to inhibition of oxidative phosphorylation *via* ATPase.

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