INHIBITION OF PURIFIED MITOCHONDRIAL ATPase (F_1) BY BATHOPHENANTHROLINE AND RELIEF OF THE INHIBITION BY UNCOUPLERS

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SUMMARY. Low concentrations of bathophenanthroline inhibit the ATPase activity of purified beef-heart F_1. The inhibition is antagonized by ATP in a fashion consistent with the involvement of a regulatory site on the enzyme. Various uncouplers, including FCCP, S-13, TTFB, dicoumarol and 2,4-dinitrophenol, relieve the bathophenanthroline inhibition, in concentrations similar to those known to uncouple mitochondrial oxidative phosphorylation.

Bathophenanthroline* (4,7-diphenyl-1,10-phenanthroline), an iron chelator, has been shown to inhibit mitochondrial electron transport (1-5). Recently it was found (6-8) that the inhibition was prevented by uncouplers of oxidative phosphorylation in both mitochondria and submitochondrial particles. BPh was also found to inhibit membrane-bound, oligomycin-sensitive ATPase (5-7), and this inhibition likewise was prevented by uncouplers (6-7). This pattern of inhibition suggested that BPh interrupts energy transfer between the electron-transport system and the site of action of oligomycin. However, the possibility could not be eliminated that the observed effects occurred on both sides of the oligomycin block. To test this possibility, the effects of BPh and uncouplers on the soluble, oligomycin-insensitive mitochondrial ATPase, F_1, were tested. The somewhat unexpected finding was made that BPh is a potent inhibitor of this enzyme, and that the inhibition is relieved by a variety of uncouplers in concentrations comparable to those known to uncouple mitochondrial oxidative phosphorylation. These results may be of interest from the viewpoints of both the reaction mechanism of F_1 and the mechanism of mitochondrial energy transduction in general.

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*Abbreviations: BPh, bathophenanthroline; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone; S-13, 3-chloro-3-butyl-2'-chloro-4'-nitrososalicylanilide; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

MATERIALS AND METHODS

F_1 was purified from "light" beef-heart mitochondria according to Horstman and Racker (9). The enzyme was stored at 4°C in half-saturated $(NH_4)_2SO_4$ containing 12.5 mM sucrose, 5 mM Tris-sulfate, 2 mM ATP and 1 mM EDTA, pH 7.5. For assay, an aliquot of the suspension was centrifuged and the sediment was dissolved in about 0.7 ml/mg protein of a medium containing 0.25 M sucrose, 25 mM Tris-sulfate and 2 mM EDTA, pH 7.5, and kept at room temperature. ATPase activity was assayed at 25°C by coupling the reaction to the pyruvate kinase and lactate dehydrogenase systems and measuring NADH oxidation at 340 nm (10); or, occasionally, by omitting the ATP-regenerating system and measuring the amount of P_i liberated by the modified Martin and Doty method (11). ATPase activity was between 30 and 70 μmoles/min./mg protein. Gel electrophoresis on SDS polyacrylamide (12) showed bands corresponding to known subunits of F_1 (13). The preparations contained appr. 14 nmoles adenine nucleotide and 2.5 μg phospholipid phosphorus per mg protein, as determined in 7 % $HClO_4$ extracts and residues, respectively. Phospholipid (14,15) and protein (16) were estimated by the procedures indicated. ATPase-inhibitor protein was purified and assayed as described by Horstman and Racker (9).

RESULTS

As shown in Fig.1, 5 μM BPh caused an immediate, nearly complete inhibition of the ATPase activity of purified beef-heart F_1. The inhibition was independent of the assay method used. At partially inhibitory BPh concent-

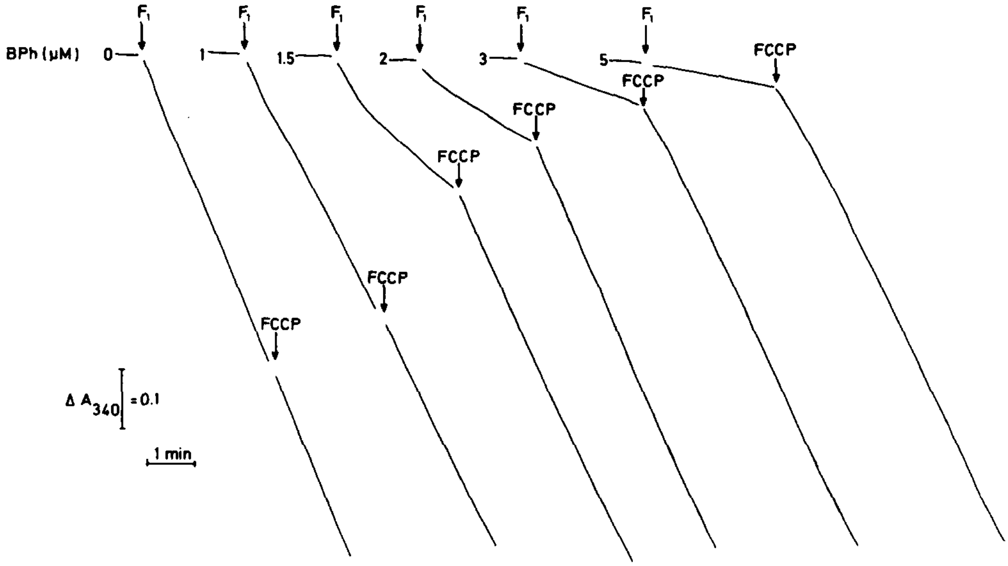

Fig. 1. Effects of BPh and FCCP on the ATPase activity of F_1
The assay medium contained 25 mM Tris-acetate, pH 7.5, 30 mM potassium acetate, 3 mM magnesium acetate, 0.18 mM NADH, 15 μg lactate dehydrogenase, 20 μg pyruvate kinase, 1 mM phosphoenolpyruvate, 2.7 mM ATP, and indicated concentrations of BPh from a stock solution of 3 mM in ethanol. The reaction was started by the addition of 3 μg F_1 protein. 1 μM FCCP was added as indicated. Total volume was 3 ml.

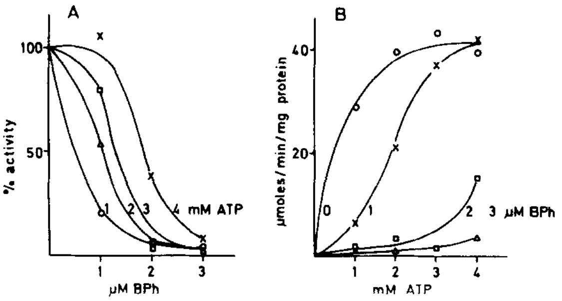

Fig. 2. Effects of BPh and ATP concentrations on the ATPase activity of F_1. Activities were measured as in Fig. 1, except that the ATP concentration was varied as indicated. The specific activities for "100 %" activity in A are the values without BPh in B.

rations the inhibition developed more slowly. 1 µM FCCP, which had no effect in the absence of BPh, relieved the inhibition.

A plot of ATPase activity vs BPh concentration was sigmoidal (Fig.2A). However, the position of the inflexion point was dependent on the ATP concentration, and the relationship tended to become hyperbolic as the latter was decreased. Conversely, the dependence of ATPase activity on the ATP concentration was hyperbolic in the absence of BPh and became sigmoidal with increasing BPh concentrations (Fig.2B). The ATPase activity was virtually linear with F_1 concentration between 0.17 and 2.5 µg protein per ml assay medium in both the absence and the presence of partially inhibitory concentrations of BPh.

Other known uncouplers such as S-13, TTFB, dicoumarol and DNP also relieved the BPh inhibition of F_1-ATPase activity (Fig.3). The effective concentrations were 0.1-3 µM for S-13, dicoumarol, FCCP and TTFB, and 10-30 µM for DNP. The uncouplers had little effect on F_1 in the absence of BPh (cf. Fig.3).

Certain ionophores, such as gramicidin and nigericin, also relieved the BPh inhibition of F_1-ATPase activity, at concentrations of 0.1-0.2 µM. On the other hand, valinomycin had little effect at 0.1-10 µM. It also did not enhance the effect of nigericin. The dependence of the nigericin effect on K^+ has not yet been determined. The BPh inhibition of F_1-ATPase was also relieved by relatively high concentrations of oligomycin (10 μM) and antimycin (3 μM) but not rotenone (5 μM). Both these compounds and the ionophores had little or no effect on F_1 in the absence of BPh. Triton X-100 (0.01 %) relieved the BPh inhibition of F_1-ATPase activity; however, in the absence of BPh 0.005 % Triton X-100 enhanced the ATPase activity 2.5-fold. Bovine serum albumin (0.02 %) relieved about 50 % of the BPh inhibition of F_1-ATPase activity without affecting the activity in the absence of BPh.

The F_1-ATPase activity was not inhibited by other iron chelators such as 1,10-phenanthroline (25 μM), 2,9-dimethyl-1,10-phenanthroline (25 μM), bathophenanthroline sulfonate (40 μM), 8-hydroxy-quinoline (33 μM), thenoyltrifluoroacetone (6.7 μM) or inorganic pyrophosphate (0.1 mM). The Fe^{2+}-BPh chelate inhibited F_1, and FCCP relieved the inhibition. An iron analysis (17) designed to detect less than 2 ng Fe in 95 μg protein revealed no iron in F_1.

FCCP did not relieve the effects of other known inhibitors of F_1 such as azide (10), aurovertin (18-20) or the mitochondrial ATPase-inhibitor protein (21). The ATPase activity remaining in the presence of partially inhibitory concentrations of these compounds could be further inhibited by BPh in a FCCP-relievable manner. BPh or FCCP, alone or in combination, did not affect the F_1-induced aurovertin fluorescence (19,20,22).

DISCUSSION

The results presented in this paper reveal some novel features of the mitochondrial ATPase F_1 that may have several important implications.

First, the high sensitivity of the purified beef-heart F_1 to BPh suggests that the enzyme might contain a metal that binds the inhibitor. The presence of Fe could be eliminated by direct analysis. A preliminary test also seems to eliminate the occurrence of Cu. An analysis of F_1 for other metals that are chelated by phenanthrolines, such as Mn, Co, and Zn, is in progress. The fact that the iron chelate of BPh was also inhibitory suggests the possibility that the inhibition is not due to metal chelation. However, it may be argued that an enzyme-bound metal efficiently competes with Fe in the added chelate. Whether or not a metal chelation is involved, it is obvious that the effect is highly specific for BPh and is not shared even by closely related compounds.

A second point of interest concerns the kinetics of the BPh inhibition. From the data in Fig.2 it appears that ATP counteracts the BPh inhibition, and, conversely, that BPh lowers the affinity of the enzyme for ATP. In both cases hyperbolic kinetics became sigmoidal. F_1 contains multiple binding-sites for adenine nucleotides (23-25) and some of these may have a regulatory function (23). It is possible that BPh inhibits F_1 by displacing ATP from a regulatory site. Studies of the binding of BPh to F_1 and its subunits, and of consequent changes in the binding patterns of adenine nucleotides, will be helpful in further clarifying this question. Whatever its precise mechanism, the BPh inhibition clearly differs kinetically from those of previously known F_1 inhibitors such as the purely competitive inhibitions by ADP (26) and imido-ATP (27, 28), the uncompetitive inhibition by aurovertin (19), or the noncompetitive inhibition by the ATPase-inhibitor protein (29,30).

A further important difference between the effects of BPh and other F_1 inhibitors is that the BPh inhibition can be reversed by uncouplers. All the uncouplers tested (cf. Fig.3) were effective in concentrations comparable to those known to uncouple oxidative phosphorylation and stimulate ATPase in mitochondria and submitochondrial particles (31-33). DNP has been shown to stimulate purified F_1 in the absence or presence of partially inhibitory concentrations of azide (10). However, this effect occurred at a much higher DNP concentration (0.5 mM) (cf. ref.10) than that required for the relief of BPh inhibition (cf. Fig.3), and was not duplicated by FCCP, but rather seems to be related to a general effect of phenols on F_1, unrelated to their capacities as uncouplers (34). The BPh inhibition of F_1 was also relieved by certain compounds, which are not known as bona fide uncouplers, but some of which - e.g. antimycin (31) - are known to act as uncouplers in high concentration.

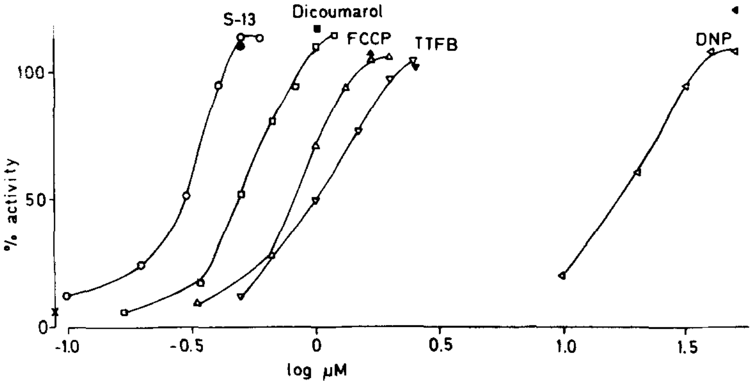

Fig. 3. Effects of uncouplers on the ATPase activity of F_1 in the presence and absence of BPh
Activities were measured as in Fig. 1. The specific activity of F_1 in the absence of BPh and uncouplers was 37 μmoles ATP/min./mg prot. Assays indicated by open symbols contained 3 μM BPh. In assays represented by the solid symbols no BPh was present. The symbol x on the ordinate indicates the ATPase activity at 3 μM BPh in the absence of uncoupler.

How do uncouplers relieve the BPh inhibition of F_1? That the effect would be due to a direct interaction of the uncoupler with BPh appears unlikely on chemical grounds and is also made improbable by the fact that certain uncouplers - e.g. S-13 - relieve the inhibition at molar amounts 5-10 times lower than the amount of BPh added (cf. Fig.3). It thus appears that the uncouplers interact with the BPh-inhibited enzyme. The uncouplers clearly could not be acting on F_1 in the same way as they are proposed to act on membrane vesicles according to the chemiosmotic hypothesis (35), since the enzyme preparation lacked the necessary amount of phospholipids to form vesicles. Indeed, to our knowledge these results represent the first instance of an effect of low concentrations of uncouplers on an isolated, soluble mitochondrial energy-transfer enzyme.

The facts that oligomycin inhibits uncoupler-stimulated mitochondrial ATPase (36), and that oligomycin-induced respiratory control is abolished by uncouplers in F_1-depleted submitochondrial particles (37-39), make it highly unlikely that F_1 is the site of action of uncouplers on mitochondrial energy transduction. However, the bathophenanthroline-inhibited F_1 may be an interesting model of an energy-transducing enzyme that can be brought into an inhibited state by binding to a specific ligand and reactivated by uncouplers. Such a mechanism of uncoupler action has, in principle, been proposed by chemical hypotheses of oxidative phosphorylation (40-44) and may equally well be applicable to more recent ideas concerning energy conservation through conformational coupling (45-49).

Added note: (February 25, 1975)
After submission of the manuscript, it was detected that the iron analysis mentioned in the Results was in error. Thus, the presence of iron in F_1 cannot be eliminated at this time.

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