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UNCOUPLER-REVERSIBLE INHIBITION OF MITOCHONDRIAL ATPase BY METAL CHELATES OF BATHOPHENANTHROLINE

II. COMPARISON WITH OTHER INHIBITORS

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Key words: F_1-ATPase; Bathophenanthroline-metal chelate; Uncoupler-reversible inhibition; Aurovertin; (Beef heart mitochondria)

(1) Trisbathophenanthroline-Fe^{2+}(BPh_3Fe^{2+}) alters the hyperbolic relationship between concentration of ATP and reaction velocity of F_1-ATPase to sigmoidal, with a simultaneous decrease in maximal velocity. (2) BPh_3Fe^{2+} binds to the β-subunit of F_1 and competes with the binding of aurovertin. The reversal of this effect by uncouplers in enhanced by ADP and diminished by ATP. BPh_3Fe^{2+} also changes the hyperbolic concentration dependence of aurovertin binding to sigmoidal. (3) BPh_3Fe^{2+} stabilizes F_1 against cold inactivation and cold dissociation in an uncoupler-reversible manner. (4) BPh_3Fe^{2+} efficiently protects F_1 against the light-induced inactivation occurring in the presence of Rose Bengal, and the effect is reversed by uncouplers. (5) The results are discussed in relation to the reaction mechanism of F_1-ATPase and other enzymes catalyzing the reversible hydrolysis of pyrophosphate bonds.

Introduction

BPh_3Fe^{2+} and other octahedral BPh_3-metal chelates have been shown to inhibit soluble mitochondrial F_1-ATPase in an uncoupler-reversible manner [1–4]. Data presented in this paper relate to the influence of BPh_3Fe^{2+} on the interaction of F_1 with its substrate and various inhibitors, in particlular aurovertin [5,6], which forms a fluorescent complex with F_1 [7] and its isolated β–subunit [8–10]. The protection of F_1 by BPh_3Fe^{2+} against the inactivation occurring upon cold exposure and irradiation in the presence of Rose Bengal [11] is also described. The results will be discussed in relation to the reaction mechanism of F_1 and other enzymes catalyzing the hydrolysis of pyrophosphate bonds.

Abbreviations: BPh, bathophenanthroline (4,7-diphenyl-1,10-phenanthroline); FCCP, carbonyl cyanide *p*-trifluorometoxyphenylhydrazone; TTFB, 4,5,6,7-tetrachlorotrifluoromethylbenzimidazole; 1799, 2,6-dihydroxy-1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptan-4-one. SDS, sodium dodecyl sulfate.

Materials and Methods

F_1-ATPase. Beef heart mitochondrial F_1-ATPase was prepared by using the method of Horstman and Racker [12]. F_1 was stored and its activity routinely measured as described in Ref. 4. Molarity of F_1 is given assuming a molecular weight of 360 000 and 100% purity.

β-Subunit of F_1. The β-subunit of F_1 was isolated according to the method of Stutterheim et al. [13], except that beef heart mitochondrial F_1, and not yeast F_1, was used as starting material. Molarity of the β-subunit is given assuming a molecular weight of 50 000 and 100% purity.

Aurovertin fluorescence. Aurovertin was obtained as a kind gift from Dr. Brian Beechey, Sittingbourne Research Centre, Sittingbourne, Kent, U.K. The con-

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centration of aurovertin dissolved in ethanol was determined spectrophotometrically at 368 nm using an absorption coefficient of 28.5 $mM^{-1} \cdot cm^{-1}$ [8]. Fluorescence measurements were made at 3°C with an Aminco-Bowman spectrophotofluorometer, with 368 nm as excitation wavelength and 470 nm as emission wavelength, in a medium containing 25 mM Tris-acetate (pH 7.6).

Polyacrylamide gel electrophoresis. Electrophoresis of native and cold-treated F_1 was performed in 6% polyacrylamide gels containing 0.16% *N,N'*-bismethyleneacrylamide, 0.4 M Tris-HCl (pH 7.5) and 43.5% glycerol as described by Hundal and Ernster [14].

Results

Dependence of BPh_3Fe^{2+} inhibition of ATPase activity on ATP and ADP concentrations

In the concentration range 0.2–4 mM, the reaction velocity of soluble F_1-ATPase was hyperbolic as a function of ATP concentration, with a K_m value of 1.1 mM (Fig. 1). Increasing concentration of BPh_3Pe^{2+} induced an increasing degree of sigmoidicity in this relationship, with a simultanous decrease in maximal velocity (Fig. 1).

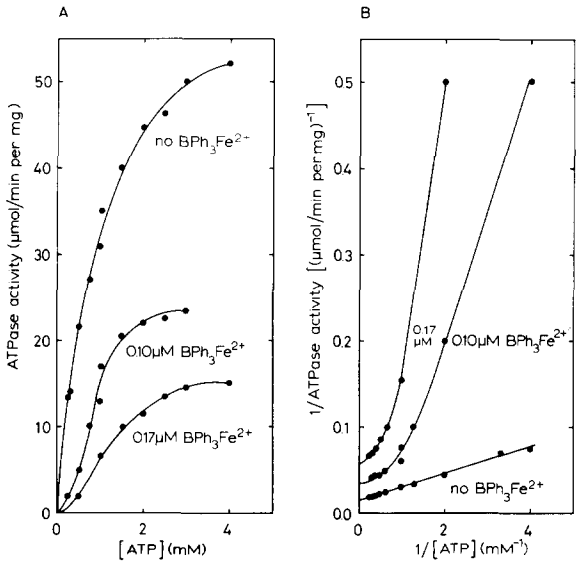

Fig. 1. Relationship between ATPase activity of F_1 and ATP concentration at varying concentrations of Ph_3Fe^{2+}. Mg^{2+} was added at a concentration equal to that of ATP.

ADP, which is a competitive inhibitor of F_1-ATPase with respect to ATP [15], had no influence on the BPh_3Fe^{2+} inhibition (not shown); this was tested by measuring the ATPase activity as H^+ formation in the absence of an ATP-regenerating system. The inhibition of F_1-ATPase by BPh_3Fe^{2+} also remained unaltered after removal of the bulk of bound adenine nucleotides by treating the enzyme with trypsin and Sephadex as described by Leimgruber and Senior [16].

Effect of BPh_3Fe^{2+} on the interaction of F_1 with aurovertin

Aurovertin, an inhibitor of mitochondrial oxidative phosphorylation [17,18] and ATPase [5,6], has been shown to interact with F_1, forming a highly fluorescent complex [7]. As shown in Fig. 2A, BPh_3Fe^{2+} inhibited the F_1-induced increase in aurovertin fluorescence, and this effect was counteracted by the uncoupler FCCP (Fig. 2A). FCCP alone exhibited no fluorescence and had a slight quenching effect on the fluorescence of the F_1-aurovertin complex. Aurovertin fluorescence in the absence of F_1 was unaffected by BPh_3Fe^{2+} (Fig. 2B).

Fig. 3 shows the effects of varying concentrations of BPh_3Fe^{2+} and FCCP in abolishing and restoring, respectively, the F_1-induced aurovertin fluorescence. This experiment was performed at a limiting concen-

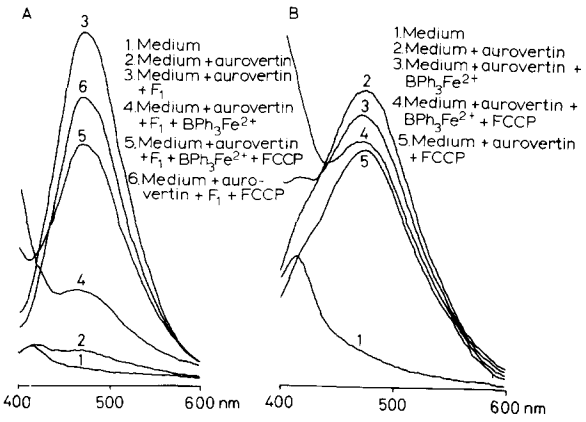

Fig. 2. Effects of BPh_3Fe^{2+} and ATP on the fluorescence of aurovertin in the presence of F_1. Conditions in A: 1 μM aurovertin, 0.14 μM F_1, 2.5 μM BPh_3Fe^{2+}, 8.3 μM FCCP. Conditions in B: 5 μM aurovertin, 2.5 μM BPh_3Fe^{2+}, 8.3 μM FCCP. The scale in B is expanded 3-fold as compared to that in A.

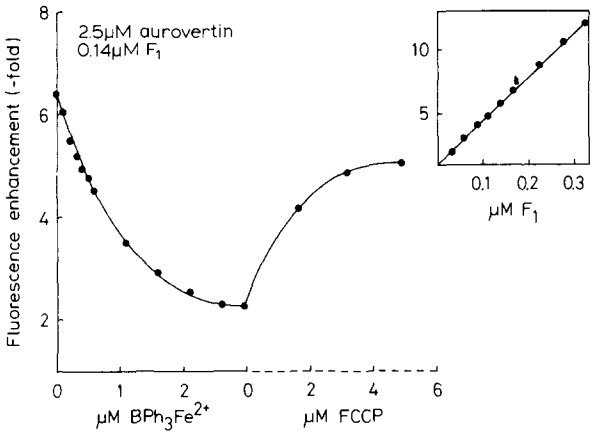

Fig. 3. Effects of varying concentrations of BPh_3Fe^{2+} and FCCP in abolishing and restoring, respectively, the F_1-induced aruovertin fluorescence.

tration of F_1 in relation to aurovertin, under which conditions the fluorescence enhancement was linear with respect to F_1concentration (see inset of Fig. 3). When using higher concentrations of F_1 this relationship became hyperbolic (Fig. 4), with an extrapolated fluorescence enhancement of 60-fold at infinite F_1 concentration. This value is in good agreement with that reported by Chang and Penefsky [19] but somewhat lower than that recently found by Muller et al. [20]. When a partially inhibitory concentration of BPh_3Fe^{2+} was added, the hyperbolic relationship was altered and showed a sigmoidal tendency (cf. Fig. 4).

A similar relationship was observed when the aurovertin concentration was varied at a fixed amount of F_1 and in the presence of different concentrations of

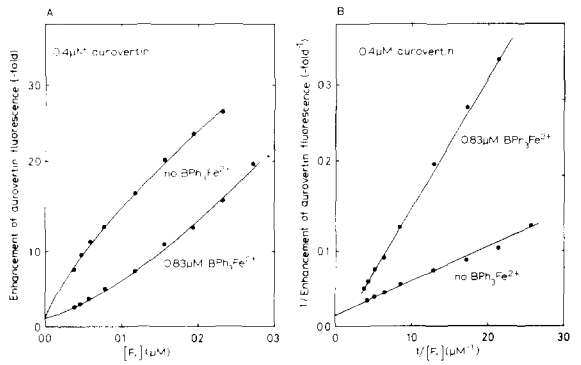

Fig. 4. Relationship between the extent of aurovertin fluorescence enhancement and F_1 concentration at varying concentrations of BPh_3Fe^{2+}.

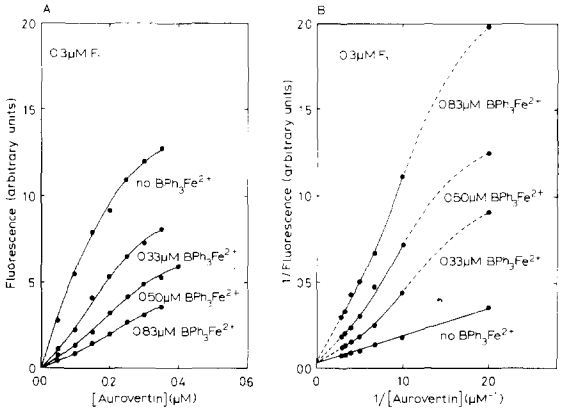

Fig. 5. Relationship between the extent of aurovertin fluorescence enhancement by F_1 and aurovertin concentration at varying concentrations of BPh_3Fe^{2+}.

BPh_3Fe^{2+} (Fig. 5). These results indicate that BPh_3Fe^{2+} induces a cooperativity between aurovertin-binding sites. In the absence of BPh_3Fe^{2+}, the aurovertin-binding sites on F_1 are independent, as reported by Verschoor et al. [8].

As shown in Fig. 6, the time course of the aurovertin fluorescence enhancement by F_1 was biphasic, and the rate and extent of a second, slow phase were influenced by ATP and ADP, in accordance with earlier observations by Chang and Penefsky [19]. It may be seen in Fig. 6 that the effect of BPh_3Fe^{2+} in abolishing the aurovertin fluorescence enhancement by F_1 concerned the rapid phase of the enhancement,

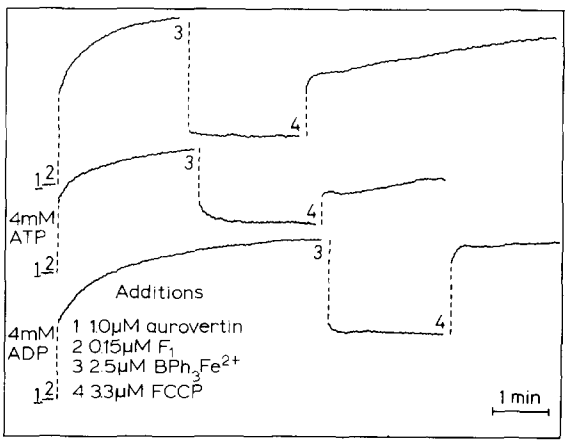

Fig. 6. Effects of ADP and ATP on the influence of BPh_3Fe^{2+} and FCCP on F_1-induced aurovertin fluorescence.

and was also itself rapid. Furthermore, the subsequent effect of FCCP in restoring the fluorescence was also rapid and its extent was influenced by ATP and ADP, being decreased by the former and increased by the latter. In the presence of ATP or in the absence of added ADP and ATP, the effect of FCCP consisted of an incomplete rapid phase followed by a slow phase.

Table I summarizes data which show that the effect of BPh_3Fe^{2+} in abolishing the F_1-induced aurovertin fluorescence enhancement was not shared by the Fe^{2+} trichelates of *o*-phenanthroline and bathophenanthrolinesulfonate. The restoration of the aurovertin fluorescence by FCCP was duplicated by other uncouplers including 1799, dicoumarol and TTFB. These experiments were performed with limiting concentrations of F_1 in relation to aurovertin, i.e., within the linear range of the aurovertin response vs. F_1 concentration. The results show that these effects of BPh_3Fe^{2+} and uncouplers follow the same pattern as the inhibition of F_1-ATPase activity [2–4].

TABLE I

EFFECTS OF VARIOUS CHELATES AND UNCOUPLERS ON THE FLUORESCENCE OF AUROVERTIN IN THE PRESENCE OF F_1

All samples contained 1 μM aurovertin and 0.13 μM F_1. oPh, *o*-phenanthroline; BPhdS, bathophenanthrolinedisulfonate.

Expt. No.	Additions	Aurovertin fluorescence enhancement by F_1 (-fold)
1	–	17.6
	BPh_3Fe^{2+}(2.5 μM)	4.1
	FCCP (8.3 μM)	14.3
	BPh_3Fe^{2+}+ FCCP	11.3
2	–	18.5
	BPh_3Fe^{2+} (2.5 μM)	4.4
	1799 (120 μM)	17.8
	BPh_3Fe^{2+} + 1799	14.8
3	–	18.0
	BPh_3Fe^{2+}(2.5 μM)	3.9
	Dicoumarol (8.3 μM)	17.6
	BPh_3Fe^{2+}+ dicoumarol	15.6
4	–	17.4
	BPh_3Fe^{2+}(2.5 μM)	4.4
	TTFB (8.3 μM)	17.3
	BPh_3Fe^{2+}+ TTFB	15.0
5	–	17.1
	oPh_3Fe^{2+} (4.2 μM)	14.8
	oPh_3Fe^{2+} + FCCP (8.3 μM)	11.8
6	–	16.3
	$BPhdS_3Fe^{2+}$ (2.5 μM)	13.8
	$BPhdS_3Fe^{2+}$ + FCCP (8.3 μM)	10.9

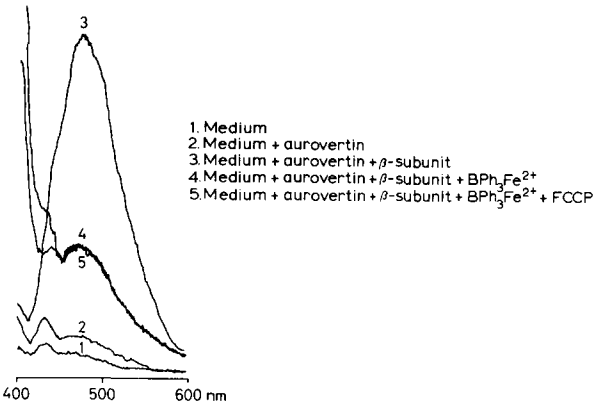

Fig. 7. Effects of BPh_3Fe^{2+} and FCCP on the aurovertin fluorescence in the prescence of the isolated β-subunit of F_1. Conditions: 1 μM aurovertin, 0.5 μM β-subunit, 5 μM BPh_3Fe^{2+}, 5 μM FCCP.

It has been shown [8–10] that the isolated β-subunit of F_1 enhances aurovertin fluorescence. The results in Fig. 7 confirm this finding and show that BPh_3Fe^{2+} abolishes this effect. In contrast to the results with the whole F_1, however, the BPh_3Fe^{2+} effect as observed with the isolated β-subunit is not reversed by FCCP. This lack of uncoupler effect may be due to the fact that, in order to respond to aurovertin, the β-subunit has to be prepared in the presence of ATP, in agreement with a report by Stutterheim et al. [13]. As shown in Fig. 6, the FCCP effect in restoring aurovertin fluorescence was counteracted by ATP (cf. Fig. 6). It is conceivable that this effect is even more pronounced with the isolated β-subunit. Added ADP had no effect in promoting an FCCP-induced restoration of aurovertin fluorescence with the isolated β-subunit.

Aurovertin inhibited the ATPase activity of F_1 to a maximal extent of about 75% (Fig. 8), which is consistent with the conclusion that aurovertin converts F_1 into a less active ATPase. This inhibition was not altered by BPh_3Fe^{2+} + FCCP, suggesting that F_1 and the $F_1BPh_3Fe^{2+}$-FCCP complex respond equally to aurovertin. When bPh_3Fe^{2+} was added to the maxi-

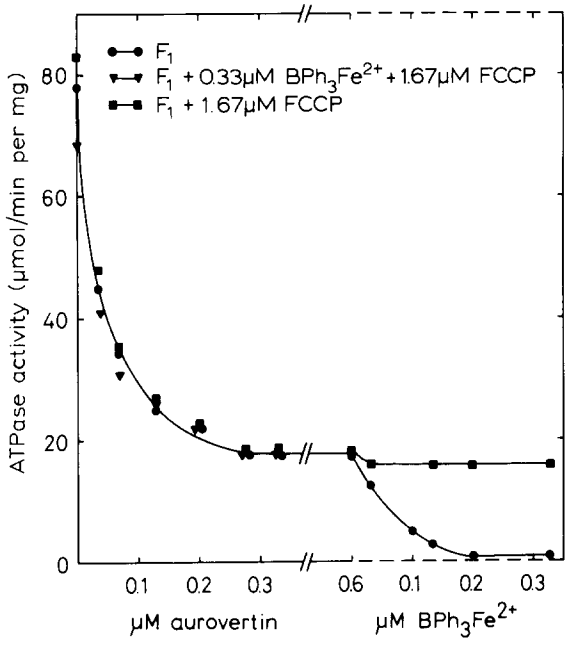

Fig. 8. Effects of BPh_3Fe^{2+} and FCCP on the inhibition of F_1-ATPase activity by aurovertin.

aurovertin. As expected, when BPh_3Fe^{2} was added to the maximally aurovertin-inhibited F_1, it further inhibited the enzyme, and this inhibition was relieved by FCCP.

Effect of BPh_3Fe^{2+} on the cold lability of F_1-ATPase

As shown in Fig. 9, BPh_3Fe^{2+} efficiently protected F_1-ATPase from cold inactivation. ATPase activity after cold exposure in the presence of BPh_3Fe^{2+} was measured following addition of the uncoupler FCCP to relieve BPh_3Fe^{2+} inhibition. When FCCP was present during cold exposure, it abolished the protective effect of BPh_3Fe^{2+}. FCCP itself had no significant effect on the cold sensitivity of F_1.

A striking protection of F_1 against cold dissociation can be demonstrated by polyacrylamide gel electrophoresis under non-denaturating conditions (in the absence of SDS). As shown elsewhere [14,21], cold exposure resulted in dissociation from the enzyme of a limited number of α- and/or β-subunits. This effect, which accompanies the release of bound adenine nucleotides from F_1, was efficiently prevented by BPh_3Fe^{2+} (Fig. 10). Again, as expected, uncouplers such as FCCP abolished the BPh_3Fe^{2+} effect.

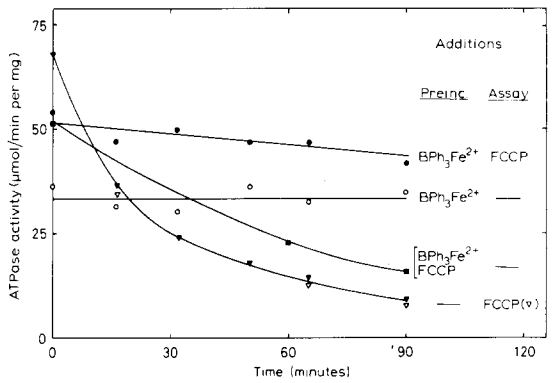

Fig. 9. Effects of bPh_3Fe^{2+} and FCCP on cold inactivation of F_1. 0.04 mg F_1 dissolved in 1 ml 0.25 M sucrose, 10 mM Tris-H_2SO_4 (pH 7.5), 2 mM EDTA was preincubated at 0°C. Aliquots of 50 μl were removed at different time intervals and ATPase activity was assayed at 30°C. When indicated, 1.7 μM FCCP was added to the assay medium.

Effect of BPh_3Fe^{2+} on the inhibition of F_1-ATPase activity by irradiation in the presence of Rose Bengal

Godinot et al. [11] have shown that photooxidation of F_1 in the presence of Rose Bengal leads to an inactivation of the enzyme. As shown in Fig. 11, BPh_3Fe^{2+} efficiently prevented the inactivation. This effect of BPh_3Fe^{2+} was counteracted by FCCP.

F_1 which was inhibited either by 4-chloro-7-nitrobenzofurazan or by photooxidation in the presence of Rose Bengal retained its ability to enhance aurovertin fluorescence. BPh_3Fe^{2+} abolished this fluorescence in an uncoupler-reversible manner. As already

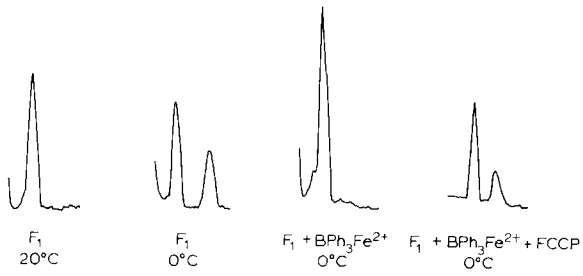

Fig. 10. Effects of BPh_3Fe^{2+} and FCCP on cold dissociation of F_1. Scanning of Coomassie blue-stained 6% polyacrylamide gels (containing no SDS) of F_1 treated in the following manner: 0.05 mg F_1 dissolved in 0.1 ml 0.25 M sucrose, 10 mM Tris-H_2SO_4 (pH 8.0), 0.25 mM EDTA was incubated at 0 or 20°C for 14 h, when indicated, in the presence of 15 μM BPh_3Fe^{2+} and 20 μM FCCP. Migration was from left to right.

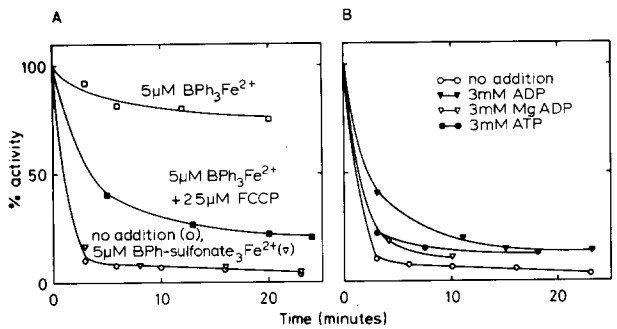

Fig. 11. Effects of BPh_3Fe^{2+} and FCCP on photooxidation of F_1 in the presence of Rose Bengal. 0.02 mg F_1 was incubated in 0.2 ml of 50 mM triethanolamine hydrochloride (pH 8.5), 100 mM KCl, 1 μM Rose Bengal and the additions indications indicated, in a small test-tube immersed in a large volume of water kept at room temperature. A 250 W slide projector was used as light source, the lens of the projector placed 10 cm from the sample and focussed to give the most concentrated light possible. Control samples containing the same amount of F_1 and Rose Bengal, but kept in the dark, or samples containing F_1 exposed to light in the absence of Rose Bengal showed no loss of activity.

reported [4], BPh_3Fe^{2+} and FCCP did not alter the inhibition of F_1 by 4-chloro-7-nitrobenzofurazan.

Discussion

The present data support the conclution that BPh-metal chelates inhibit F_1 by binding to its β-subunit, which is believed to contain the catalytic site of the enzyme [22,23]. The binding site of the BPh-metal chelate on F_1 probably is in close interaction with the aurovertin-binding site, as indicated by the converging double-reciprocal plots in Fig. 5; identity of the BPh_3Fe^{2+}- and aurovertin-binding sites is unlikely in view of the finding that uncouplers restore aurovertin fluorescence without removing BPh_3Fe^{2+} from the enzyme (Ref. 4, Fig. 9).

It has been shown [8,13] that one molecule of F_1 binds two molecules of aurovertin, one to each of its β-subunits, whereas the third β-subunit does not react with aurovertin even after dissociation of the enzyme with LiCl. It has also been shown [8,13] that the binding of aurovertin to the two β-subunits is non-cooperative. In contrast, BPh-metal chelates seem to bind to all three β-subunits [2,3], and to induce cooperativity between the aurovertin-binding sites of the enzyme (Fig. 5). Likewise, BPh-metal chelates seem to render the kinetics of F_1 cooperative with respect to ATP (Fig. 1).

Like several other inhibitors, including 4-chloro-7-nitrobenzofurazan [21] and the ATPase inhibitor protein [24], BPh-metal chelates render F_1 cold stable. In fact, BPh-metal chelates appear to be very efficient in preventing the dissociation of F_1 occurring upon cold exposure (Fig. 10). In view of the multiple reactive groups of these chelates it is tempting to speculate that this stabilization may involve a cross-linking of subunits of the enzyme. Whether BPh-metal chelates prevent the release of bound adenine nucleotides of F_1, known to accompany cold dissociation [25], remains to be investigated.

A striking effect of BPh-metal chelates, not shared by other known inhibitors, is its protective effect against photooxidation of F_1 as induced by Rose Bengal. This finding suggests that BPh-metal chelates protect some amino acid residue(s) essential for catalysis. ATP and ADP provide a relatively weak protection against Rose Bengal-induced photooxidation, suggesting that the residue(s) in question may not be the ATP- and ADP-binding catalytic sites.

We have reported elsewhere [26] that BPh-metal ion chelates inhibit a number of additional ATPase and other pyrophosphate-bond hydrolyzing enzymes, including chloroplast and bacterial F_1-ATPase, cell membrane $(Na^+ + K^+)$-ATPase, sarcoplasmic Ca^{2+}-ATPase, actomyosin-ATPase, microsomal nucleoside di-and triphosphatases as well as yeast and bacterial pyrophosphatases; in all cases, except yeast pyrophosphatase, the inhibition was relieved by uncouplers. Enzymes cleaving pyrophosphate bonds in a nonhydrolytic way (i.e., transferases), such as adenylate kinase, hexokinase and pyruvate kinase, as well as hydrolytic enzymes which split nonpyrophosphate bonds, including AMPase, glucose-6-phosphatase, intestinal alkaline phosphatase and muscle aldolase, were not inhibited by BPh-metal chelates. Thus, the inhibition appears to be specific for enzymes catalyzing the hydrolysis of pyrophosphate bonds and may reflect a feature in common between the catalytic mechanisms of these enzymes.

Another common feature of these enzymes seems to be that they catalyze a reversible hydrolytic cleavage of a P-O bond as revealed by a rapid oxygen exchange between P_i and water (cf. Ref. 26). It has been speculated [26] that this reaction may involve

the transport of a proton, resulting from the hydrolytic cleavage of the P-O bond in the hydrophobic center of the enzyme, to the surrounding aqueous medium. This mechanism would be similar to that involved in the hydrolytic cleavage of a C-C bond in the reaction catalyzed by muscle aldolase [27], which is inhibited by photooxidation in the presence of Rose Bengal. In that case, the proton is transported through a number of histidine residues located near the active center of the enzyme. In the present case, the proton transport may likewise involve some aromatic residues, which would serve as proton conductors between the enzyme and the hydrophilic external environment. BPh_3Fe^{2+} may exert its inhibitory effect by blocking these aromatic residues through its phenyl groups, which are essential for inhibition. The effect of uncouplers in relieving the BPh_3Fe^{2+} inhibition may be due to a mediation of protons between the hydrophobic center of the BPh_3Fe^{2+}-blocked enzyme and its aqueous environment. This proton conduction by uncouplers may be due to a displacement of the phenyl groups of BPh_3Fe^{2+} in relation to the aromatic residues of the enzyme. Such a mechanism appears conceivable in view of the earlier demonstrated nonenzymic interaction between BPh_3Fe^{2+} and various uncouplers which requires the phenyl groups of BPh_3Fe^{2+} and results in a spectral shift of interacting molecules [2,4].

In conclusion, the results presented in this and previous papers [1–4] suggest that BPh_3Fe^{2+} may be a particularly useful tool for elucidating the mechanism of pyrophosphate bond biosynthesis in oxidative and photosynthetic phosphorylation.

Acknowledgements

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