Biochimica et Biophysica Acta, 638 (1981) 345–357
Elsevier/North-Holland Biomedical Press

BBA 48174

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

I. GENERAL FEATURES

CHRISTINE CARLSSON and LARS ERNSTER

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm (Sweden)

(Received March 6th, 1981)

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

(1) Certain metal chelates of 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline, BPh) are potent inhibitors of soluble mitochondrial F_1-ATPase. (2) The BPh-metal chelate inhibition of soluble mitochondrial F_1-ATPase is relieved by uncouplers of oxidative phosphorylation. (3) The uncouplers appear to interact directly with the inhibitory chelates, forming stoichiometric adducts. (4) A complex between F_1 and bPh_3Fe^{2+}, containing 3 mol BPh_3Fe^{2+}/mol F_1, has been isolated. The enzymically inactive F_1-BPh_3Fe^{2+} complex binds uncouplers, yielding an enzymically active F_1-BPh_3Fe^{2+}-uncoupler complex.

Introduction

The mitochondrial ATPase 'coupling factor 1' (F_1) [1] is generally believed to constitute the enzyme responsible for the synthesis of ATP from ADP and P_i during electron transport. Together with similar enzymes found in chloroplasts and bacteria, the F_1-ATPase has been the subject of intensive studies over the past years (for a recent review, see Ref. 2). Through an as yet poorly understood mechanism, these enzymes are structurally and functionally linked to a proton-translocating device, F_0, across the membrane, and operate in this integrated form as reversible proton pumps capable of utilizing a trans-membrane proton gradient as the driving force for ATP synthesis.

Various inhibitors have proved to be useful tools for studying the reaction mechanism of F_1 and its interaction with F_0 [3]. In our laboratory it was found that certain metal chelates of bathophenanthroline are potent inhibitors of soluble and membrane-bound mitochondrial ATPase [4,5]. A striking feature of this inhibition, not shared by previously known ATPase inhibitors, is its reversibility by uncouplers of oxidative phosphorylation. Evidence was obtained for a direct interaction between uncouplers and the inhibitory chelates [5]. Data were also briefly reported concerning the binding of the inhibitory chelates and of uncouplers to F_1 [6].

In the present paper these effects are investigated in some detail. A comparison of the effects of BPh-metal chelates with those of other inhibitors of F_1-ATPase is the subject of the accompanying paper [7].

Materials and Methods

F_1-ATPase. F_1-ATPase was purified from beef heart mitochondria according to the method of

Abbreviations: BPh, bathophenanthroline (4,7-diphenyl-1,10-phenanthroline); DPBP, 4,4-diphenyl-2,2-bipyridine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PDT, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine; 5-PhoPh, 5-phenyl-1,10-phenanthroline; S-13, 3-chloro-3-butyl-2′-chloro-4′-nitrososalicylanilide; TMoPh, 3,4,7,8-tetramethyl-1,10-phenanthroline; TTFB, 4,5,6,7-tetrachloro-2-trifuoromethylbenzimidazole; 1799, 2,6-dihydroxy-1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptan-4-one; oPh, *o*-phenanthroline (1,10-phenanthroline); TPTZ, 2,4,6-tripyridyl-*S*-triazine.

0005-2728/81/0000–0000/$02.50 © 1981 Elsevier/North-Holland Biomedical Press

Horstman and Racker [8]. It had a specific activity of approx. 100 μmol/min per mg protein at 30°C. The enzyme was stored at 4°C in a 50% saturated $(NH_4)_2SO_4$ suspension containing 0.125 M sucrose, 50 mM Tris-H_2SO_4, 1 mM EDTA and 3 mM ATP (pH 7.6). Before use, an aliquot of the suspension was centrifuged, and the sediment was dissolved in 0.25 M sucrose, 10 mM Tris-H_2SO_4 and 2 mM EDTA (pH 7.6).

Submitochondrial particles. MgATP and EDTA particles were prepared from beef heart mitochondria as described by Lee and Ernster [9].

ATPase activity. ATPase activity was routinely assayed by coupling the reaction to the pyruvate kinase and lactate dehydrogenase systems and measuring NADH oxidation [1]. When indicated, ATPase activity was assayed directly by determining colorimetrically the amount of P_i liberated [10].

NADH oxidation. NADH oxidation was followed spectrophotometrically at 340 nm.

Oxidative phosphorylation. O_2 consumption was measured with a Clark oxygen electrode, and phosphate uptake was determined by the isotope distribution method [10].

Protein determinations. Soluble proteins was determined by the method of Lowry et al. [11] and particulate protein by the biuret method, using in both cases bovine serum albumin as standard.

Chelates. Chelates of bathophenanthroline and related compounds were prepared by dissolving the chelator in ethanol (except in the case of bathophenanthrolinesulfonate, which was dissolved in water) and mixing it with the appropriate concentration of the metal salt dissolved in water to give a final ethanol concentration of 50%.

Bathophenanthroline, bathophenanthrolinesulfonate, 5-PhoPh, DPBP, PDT and TPTZ were purchased from G. Frederick Smith Chemical Co. Other phenanthroline derivatives and BPh_3Ru^{2+} were kind gifts from Dr. David Sigman, Department of Biological Chemistry, University of California, Los Angeles.

Results

Effects of BPh-metal chelates on F_1-ATPase

Fig. 1 shows the effect of bathophenanthroline on the activity of beef heart mitochondrial F_1-ATPase in the presence of various metal ions. The strongest inhibition was obtained in the presence of Fe^{2+}, Ni^{2+} or Ru^{2+}, when these ions were added as the preformed BPh-metal chelates prepared by mixing bathophenanthroline and the metal ions in concentration ratios of 3. Under these conditions, the BPh-metal chelates are predominantly formed, as judged from the available stability constants of the corresponding *o*-phenanthroline chelates [12]. Co^{2+} and Zn^{2+}, which also form stable trichelates with bathophenanthroline, although weaker than Fe^{2+}, Ni^{2+} or Ru^{2+}, likewise enhanced the inhibition of F_1 by bathophenanthroline; however, the inhibition was weaker than in the case of Fe^{2+}, Ni^{2+} or Ru^{2+}. In all cases, the inhibition decreased when the BPh : metal ion ratio was lowered to 1, and the inhibition did not increase when the BPh : metal ion ratio was increased above 3. Mn^{2+}, which forms a weak chelate with bathophenanthroline, did not enhance the effect of the latter.

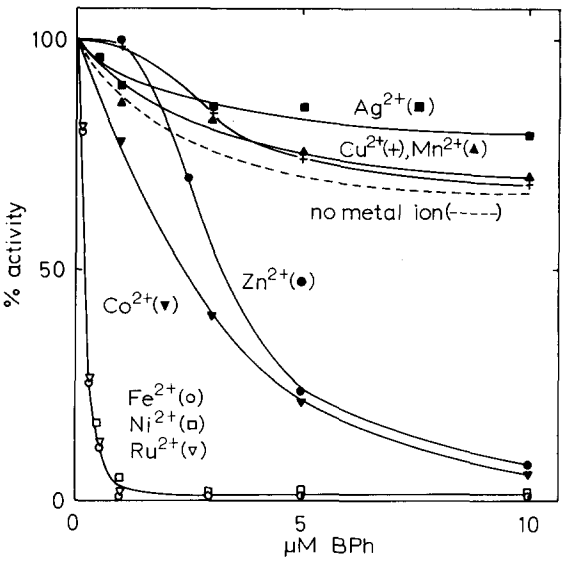


Fig. 1. Effect of bathophenanthroline on F_1-ATPase activity in the presence of various metal ions. The assay medium contained 25 mM Tris-acetate, 30 mM potassium acetate, 3 mM magnesium acetate, 0.2 mM NADH, 1 mM phospho*enol*pyruvate, 50 μg lactate dehydrogenase, 50 μg pyruvate kinase and 3 mM ATP (pH 7.6). Bathophenanthroline was added in varying concentrations as the preformed metal trichelate. The reaction was started by addition of 2.5 μg F_1 to a total volume of 3 ml.

The above-mentioned metal ions are known to

form octahedral trichelates with *o*-phenanthroline [13]. Cu^{2+} and Ag^{2+}, which also form stable trichelates with *o*-phenanthroline, but do not give rise to octahedral structures, did not enhance the bathophenanthroline inhibition of F_1. It would thus appear that the inhibition requires an octahedral BPh-metal trichelate. The weak inhibition found with bathophenantroline alone may well be due to a trace of a metal ion in the incubation medium and may also explain the earlier reported stronger inhibition [4], if one assumes that some of the reagents was contaminated by, e.g., Fe^{2+}.

Effects of other chelators

The inhibitory effect of the BPh-metal chelates on F_1-ATPase proved to be highly specific for bathophenanthroline, not being shared even by closely related phenanthrolines. Some relevant data are summarized in Table I, which compares the effects of various phenanthrolines and related compounds, all treated as the preformed Fe^{2+} trichelates (except in the case of TPTZ, which only forms an Fe^{2+} dichelate), on beef heart F_1-ATPase. The only compound tested that gave an effect comparable in strength to that of bathophenanthroline was DPBP. Omission, relocation or replacement of the phenyl groups abolished or greatly diminished the inhibitory effect, as did conversion of bathophenanthroline to the water-soluble bathophenanthrolinesulfonate. As expected, mixed chelates, e.g., those containing both bathophenanthroline and *o*-phenanthroline, had weaker effects than BPh_2Fe^{2+} (Fig. 2A). Furthermore, Fe^{2+} chelators other than bathophenanthroline could relieve the inhibitory effect of the latter (Fig. 2B).

Effects of uncouplers on BPh_3Fe^{2+}-inhibited F_1-ATPase

Fig. 3 illustrates the relief of the BPh_3Fe^{2+} inhibition of F_1-ATPase by various uncouplers. This effect was observed with all the uncouplers tested thus far, including S-13, FCCP, TTFB, dicoumarol, gramicidin D, 2,4-dinitrophenol and 1799 (Fig. 3A), and the relative efficiencies of the various substances in relieving the BPh_3Fe^{2+} inhibition of F_1 were similar to their known uncoupling activities on mitochondrial oxidative phosphorylation [14]. A good correlation between the uncoupling effect on oxidative phosphorylation and the ability to relieve the BPh_3Fe^{2+} inhibition is also found when comparing various phenols (Fig. 3B). An interesting exception is picrate, which is a poor uncoupler of oxidative phosphorylation but efficiently relieves the BPh_3Fe^{2+} inhibition of F_1-ATPase. However, as shown by Hanstein and Hatefi [15], the lack of uncoupling effect of picrate on intact mitochondria is probably due to the impermeability of the mitochondrial inner membrane to picrate.

Effects of anions on BPh_3Fe^{2+}-inhibited F_1-ATPase

Anions such as phosphate (20 mM), sulfate (150

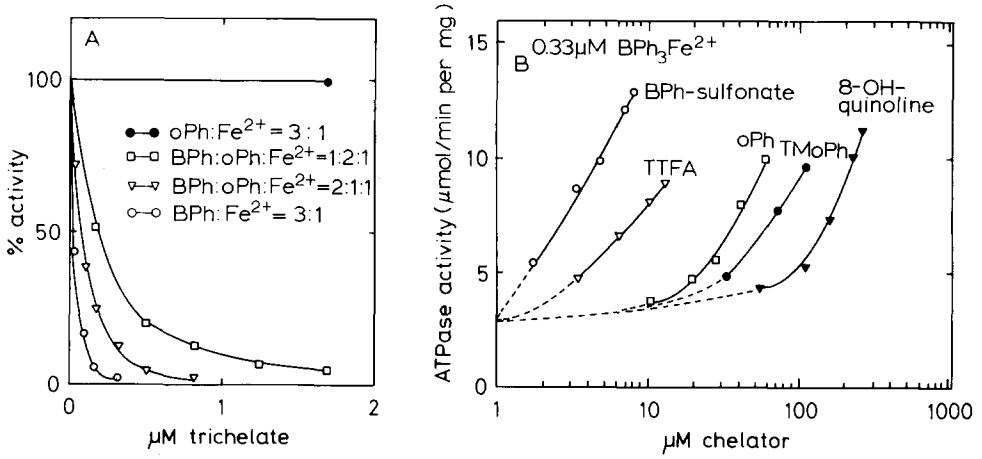


Fig. 2. Effects of other chelators on BPh_3Fe^{2+} inhibition of F_1-ATPase. Comparison of the inhibitory effects of preformed mixed chelates containing different oPh : BPh : Fe^{2+} ratios on F_1-ATPase activity (A), and the effects of other Fe^{2+} chelators on the BPh_3Fe^{2+} inhibition of F_1-ATPase activity (B). TTFA, thenoyltrifluoroacetone.

TABLE I

EFFECT OF VARIOUS CHELATES ON F_1-ATPase ACTIVITY

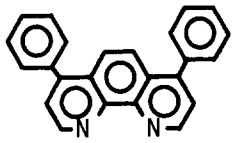
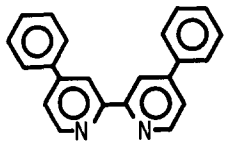
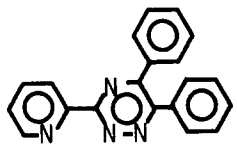
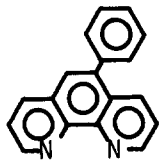
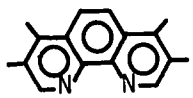
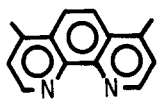
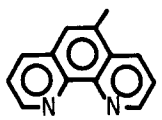
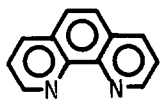
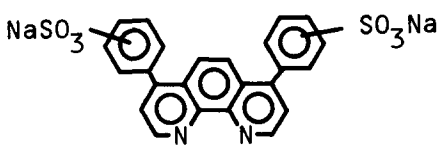
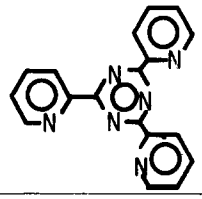
Chelator (added as Fe^{2+} trichelate) [a]		Chelate (μM)	Relative ATPase activity
BPh		0.67 3.33	0 0
DPBP		0.67 3.33	15 1
PDT		0.67 3.33	27 5
5-PhopH		0.67 3.33	67 33
TMoPh		0.67 3.33	70 43
4,7-Dimethyl-1,10-phenanthroline		0.67 3.33	96 43
5-Methyl-1,10-phenantholine		3.33	76
oPh		33 250	100 83
Bathophenanthrolinedisulfonate		33	100

TABLE I (continued)

Chelator (added as Fe^{2+} trichelate) [a]		Chelate (μM)	Relative ATPase activity
TPZT		0.67 3.33	100 100

[a] Except in the case of TPTZ, which only forms an Fe^{2+} dichelate.

mM) and sulfite (4 mM), which have been shown to activate F_1-ATPase, did not relieve the inhibition by BPh_3Fe^{2+} (cf. Ref. 5). Conversely, uncouplers did not relieve the inhibition of F_1-ATPase by azide [1], aurovertin [16], 4-chloro-7-nitrobenzofuran [17,18] or the protein inhibitor described by Pullman and Monroy [19].

Influence of pH of F_1-ATPase, its inhibition by BPh_3Fe^{2+} and reactivation by FCCP

As shown in Fig. 4, the inhibition of F_1-ATPase by BPh_3Fe^{2+} was virtually independent of the pH of the medium in the range 6.0–8.7. The relief of the inhibition by FCCP decreased in efficiency with increasing pH. The ATPase activity itself had a pH optimum at 8.6, in accordance with earlier reports [20].

Effects of uncouplers on inhibition of F_1-ATPase by various inhibitory chelates

As illustrated by the data in Table II, uncouplers, such as FCCP, relieved the inhibition of F_1-ATPase not only by BPh_3Fe^{2+} but also by BPh_3Ni^{2+} and BPh_3Ru^{2+} as well as $DPBP_3Fe^{2+}$ and PDT_3Fe^{2+}. In the case of PDT_3Fe^{2+}, which is a relatively weak inhibitor, the uncoupler effect also is weaker. In general, as shown earlier [5] and further demonstrated below, the concentration of uncoupler needed for relief of the inhibition increases with the concentration of inhibitor used.

Relationship between inhibitor and uncoupler concentrations

Fig. 5 compares titrations of the relief of BPh_3Fe^{2+}

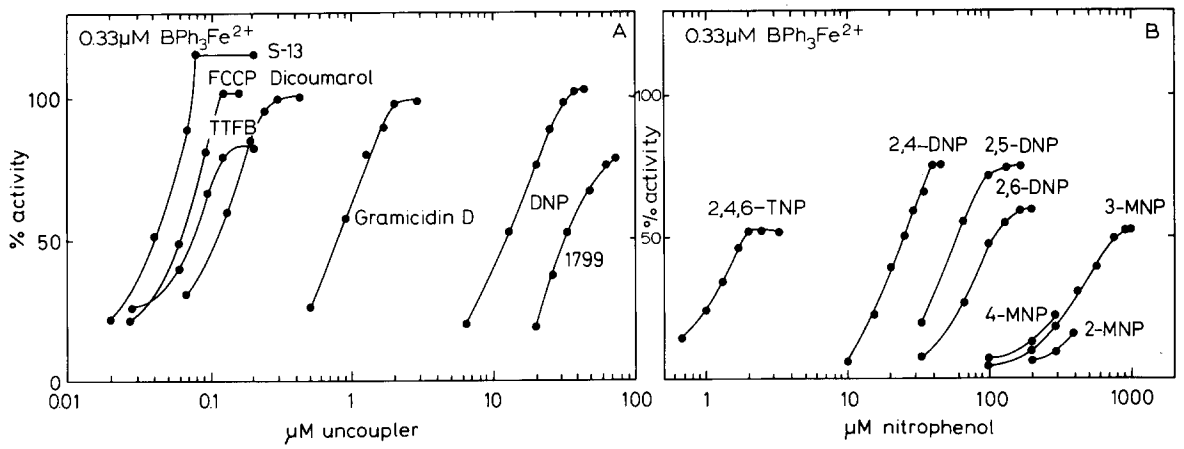


Fig. 3. Effects of uncouplers on F_1-ATPase activity inhibited by BPh_3Fe^{2+}. DNP, dinitrophenol; TNP, trinitrophenol; MNP, mononitrophenol.

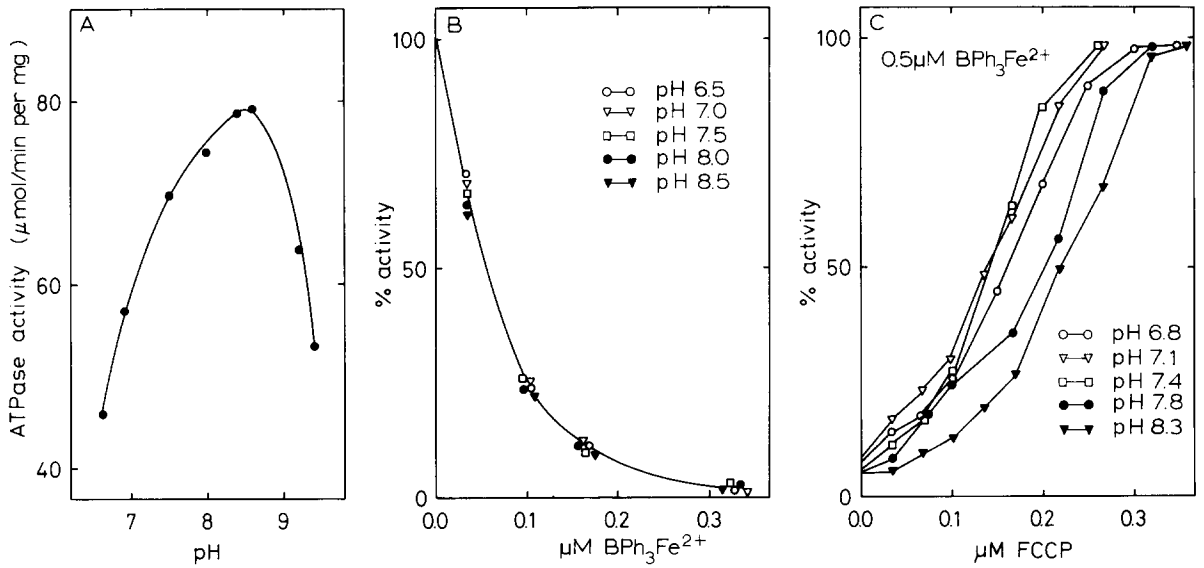


Fig. 4. Influence of pH on F_1-ATPase activity (A), its inactivation by BPh_3Fe^{2+} (B) and reactivation by FCCP (C).

inhibition of F_1-ATPase by the uncouplers S-13, FCCP and 2,4-dinitrophenol. In all these cases, the titration curves were sigmoidal when using a BPh_3Fe^{2+} concentration in excess of that needed for maximal inhibition. The sigmoidicity disappears as the BPh_3Fe^{2+} concentration is decreased so as to be just sufficient to give near-maximal inhibition. This type of effect is consistent with a direct interaction between the uncouplers and the inhibitor, resulting in an 'inactivation' of the inhibitor.

TABLE II

EFFECT OF FCCP ON THE INHIBITION OF F_1-ATPase ACTIVITY BY VARIOUS CHELATES

FCCP was added at a concentration twice as high as that of the chelate.

Chelate	Concentration (μM)	Relative ATPase activity	
		−FCCP	+FCCP
BPh_3Fe^{2+}	0.10	10	92
BPh_3Fe^{2+}	0.33	2	73
BPh_3Ru^{2+}	0.10	27	90
BPh_3Ru^{2+}	0.33	2	90
BPh_3Ni^{2+}	0.10	90	100
BPh_3Ni^{2+}	0.33	5	90
$DPBP_3Fe^{2+}$	0.10	80	93
$DPBP_3Fe^{2+}$	0.33	29	91
PDT_3Fe^{2+}	0.67	21	85
PDT_3Fe^{2+}	3.33	4	30

Interaction between inhibitory chelates and uncouplers: Spectral evidence

Direct evidence for an interaction between BPh_3Fe^{2+} and various uncouplers was obtained in the form of spectral changes that occurred when BPh_3Fe^{2+} was mixed with various uncouplers in an aqueous medium in the absence of F_1 [5]. In the experiment shown in Fig. 6A, the visible spectrum of 20 μM BPh_3Fe^{2+} was recorded in the presence of

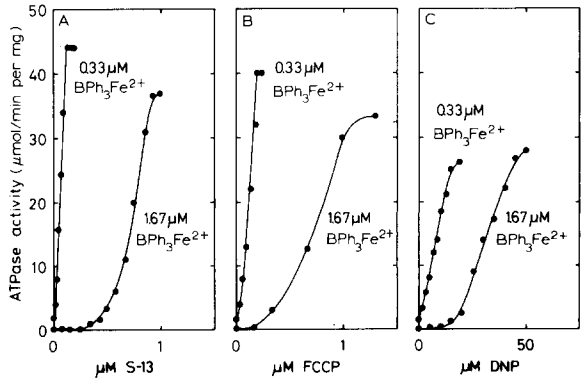


Fig. 5. Relationship between inhibitor and uncoupler concentrations. DNP, 2,4-dinitrophenol.

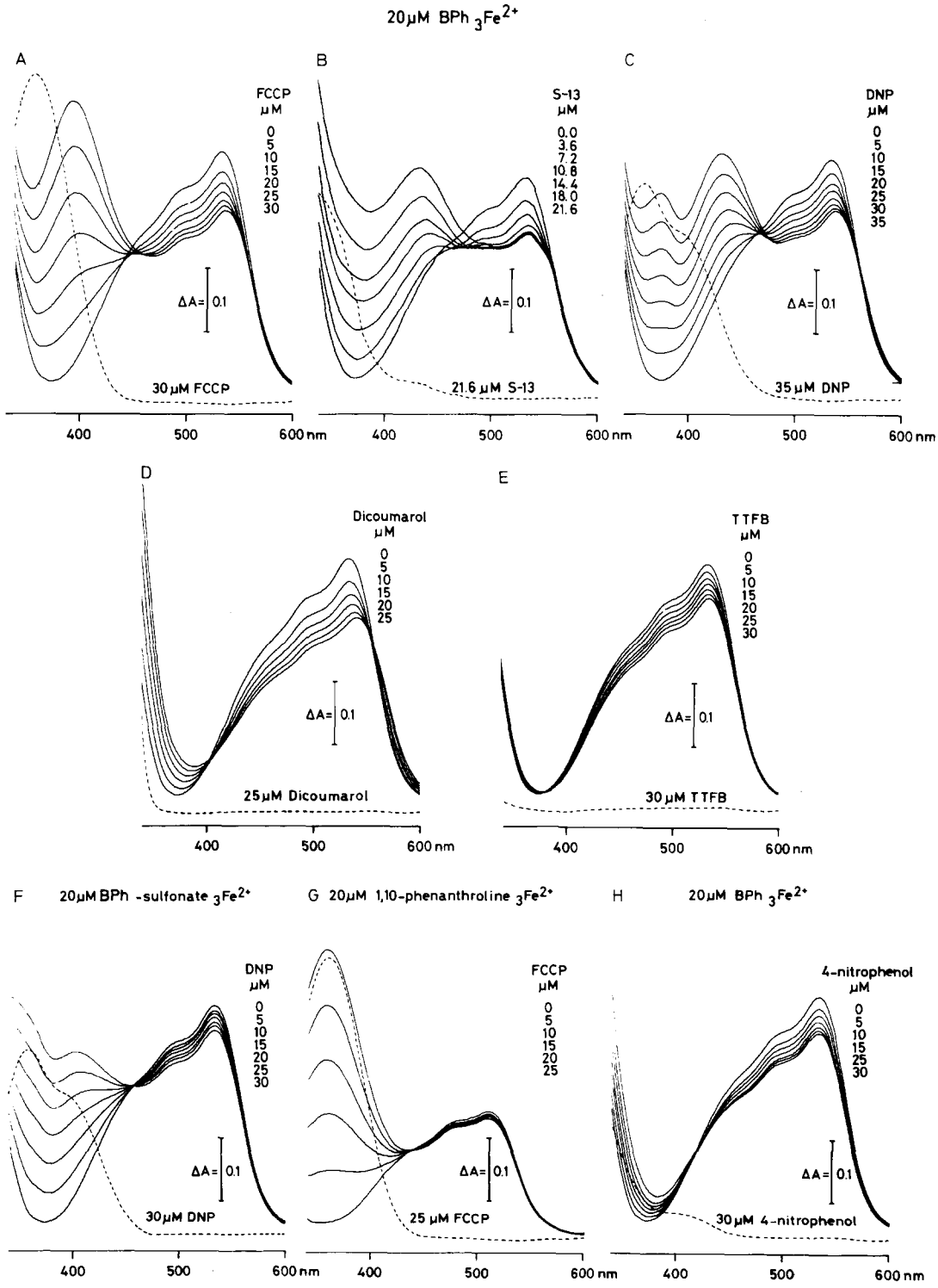


Fig. 6. Spectra of inhibitory and noninhibitory chelates in the presence of various uncouplers. DNP, 2,4-dinitrophenol.

increasing concentrations of FCCP. It may be seen that increasing concentrations of FCCP caused a progressive decrease and a slight red shift of the 535 nm absorption maximum of BPh_3Fe^{2+} which approached an asymptote when the FCCP concentration exceeded that of BPh_3Fe^{2+}. Simultaneously, there appeared a new peak at 400 nm, which increased with increasing FCCP concentration. The dotted line indicates the visible spectrum of FCCP in the absence of BPh_3Fe^{2+}. It is clear that the new peak represents a shift of the absorption spectrum of FCCP. In other words, it appears that BPh_3Fe^{2+} and FCCP interact in such a way as to cause a decrease and a red shift in the absorption maxima of both bPh_3Fe^{2+} and FCCP.

As shown in Fig. 6B–E, similar changes were found when BPh_3Fe^{2+} was mixed with other uncouplers such as S-13, 2,4-dinitrophenol, dicoumarol and TTFB, in the sense that all gave a decrease, and, with the possible exception of TTFB, a slight red shift of the absorption maximum of BPh_3Fe^{2+}. Also similar to FCCP, 2,4-dinitrophenol underwent a spectral shift, consisting of a splitting of its peak and soulder into two distinct absorption maxima at higher wavelengths, which increased with increasing 2,4-dinitrophenol concentration. The other uncouplers lack absorption in the visible region, and there appears no peak in this region in the presence of BPh_3Fe^{2+}. The occurrence of spectral changes of uncouplers in the ultraviolet region is not possible to detect in the presence of BPh_3Fe^{2+}.

It is noteworthy that changes similar to those shown in Fig. 6A–E were not found when a non-inhibitory phenanthroline-Fe^{2+} complex was mixed with an uncoupler or when BPh_3Fe^{2+} was mixed with a nonuncoupling phenol. This is illustrated in Fig. 6F–H by the combinations $BPh\text{-sulfonate}_3Fe^{2+}$-FCCP, oPh_3Fe^{2+}-2,4-dinitrophenol, and BPh_3Fe^{2+}-4-nitrophenol. In all three cases, the absorption spectrum of the phenanthroline-Fe^{2+} chelate remained unchanged, except for a slight decrease in the 535 nm peak, due to dilution. Furthermore, FCCP and 2,4-dinitrophenol underwent no spectral shift.

Separation of inhibitory chelates and their uncoupler adducts

The interaction between BPh_3Fe^{2+} and uncouplers was further studied by investigating the distribution of these compounds between water and diethyl ether. In the experiment in Fig. 7A, 50 nmol of BPh_3Fe^{2+} and varying amounts of FCCP, dissolved in a few microliters of 50 and 95% ethanol, respectively, were mixed in 5 ml of 25 mM Tris-HCl (pH 7.5) and extracted with 5 ml diethyl ether. In the absence of FCCP, all BPh_3Fe^{2+} remained in the water phase. With increasing concentrations of FCCP, an increasing proportion of BPh_3Fe^{2+} was recovered in the ether phase, reaching 100% at a molar ratio of BPh_3Fe^{2+} : FCCP of approx. 2. In all samples, except in those containing molar ratios of BPh_3Fe^{2+} : FCCP of 0.5 and 1, practically all FCCP was recovered in the ether phase. At BPh_3Fe^{2+} : FCCP molar ratios of 0.5 and 1, some FCCP was found in the water phase. These data are consistent with the interpretation that BPh_3Fe^{2+} and FCCP form a complex with a stoichiometry of BPh_3Fe^{2+} : FCCP = 2, and that the latter is less polar than either FCCP or BPh_3Fe^{2+} itself. The simplest explanation of these findings is that FCCP, which is a weak monovalent acid, neutralizes the positive charges of BPh_3Fe^{2+} and forms a strong hydrophobic complex with the latter.

Similar findings were made with another uncoupler, dicoumarol (Fig. 7B), except that dicoumarol was partly recovered in the water phase even in the absence of BPh_3Fe^{2+}. However, just as in the case of FCCP, practically all dicoumarol and BPh_3Fe^{2+} was recovered in the ether phase at a molar ratio of BPh_3Fe^{2+} : dicoumarol of 2, indicative of the formation of a nonpolar complex between the two with the above stoichiometry.

Data summarized in Table III relate to similar experiments with other chelates and uncouplers. The conclusions that can be derived from these data are as follows: (1) S-13 and BPh_3Fe^{2+} influence their mutual distribution between water and ether in a way which is consistent with the formation of a complex between the two. In contrast to the FCCP and dicoumarol complexes of BPh_3Fe^{2+}, the S-13 complex is recovered in the water phase. Its stoichiometry cannot be deduced from the data. (2) 2,4-Dinitrophenol and BPh_3Fe^{2+} influence their mutual distribution between water and ether in a way which is consistent with the formation of a complex between the two. Similar to the FCCP and dicoumarol complexes of BPh_3Fe^{2+}, the 2,4-dinitrophenol complex is less polar than BPh_3Fe^{2+} alone, but also less polar than 2,4-

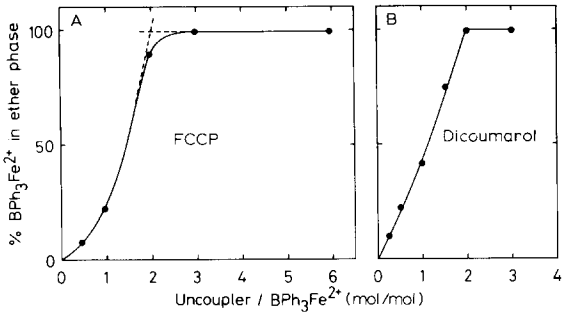


Fig. 7. Distribution of inhibitory chelates and their uncoupler adducts between water and diethyl ether. For experimental conditions see text. Visible spectra of water and ether phases were recorded.

dinitrophenol alone. The stoichiometry of the complex is not known. (3) $DPBP_3Fe^{2+}$ interacts with FCCP and dicoumarol in a fashion similar to that of BPh_3Fe^{2+}, but the stoichiometry of the complex has not been determined. (4) The following chelates and uncouplers did not influence each other's distribution between water and ether: oPh_3Fe^{2+}-FCCP, oPh_3Fe^{2+}-dicoumarol, BPh-sulfonate$_3Fe^{2+}$-FCCP, $TMoPh_3Fe^{2+}$-FCCP and 5-$PhoPh_3Fe^{2+}$-FCCP. These data are consistent with a correlation between chelates that inhibit F_1-ATPase and those capable of complex formation with uncouplers.

Binding of BPh_3Fe^{2+} to F_1

When F_1 was mixed with an excess of BPh_3Fe^{2+} and passed through a Sephadex G-25 column the enzyme was accompanied by BPh_3Fe^{2+}, as determined spectrophotometrically, in an amount that reached asymptotically a value of 3 mol bPh_3Fe^{2+}/mol F_1 when the eluate was allowed to stand at room temperature (Fig. 8). Initially, the eluate contained an additional amount of BPh_3Fe^{2+}, which probably represents unspecific, loose binding, and which dissociates into bathophenanthroline and Fe^{2+} as it is released from the enzyme; the dissociation is enhanced by EDTA [6]. The F_1-$(BPh_3Fe^{2+})_3$ complex was enzymically inactive and could be reactivated by uncouplers. Little or no binding to F_1 was obtained with oPh_3Fe^{2+} and BPh_3Fe^{2+}-sulfonate.

TABLE III

DISTRIBUTION OF VARIOUS CHELATES AND/OR UNCOUPLERS BETWEEN WATER AND DIETHYL ETHER

50 nmol of the chelate and 150 nmol of the uncoupler were used. Other conditions as described in the text.

Sample	Distribution	
	Ether phase	Water phase
BPh_3Fe^{2+}		100% BPh_3Fe^{2+}
FCCP	100% FCCP	
BPh_3Fe^{2+} + FCCP	100% BPh_3Fe^{2+} + 100% FCCP	
2,4-Dinitrophenol		100% 2,4-dinitrophenol
BPh_3Fe^{2+} + 2,4 dinitrophenol	50% BPh_3Fe^{2+} + 50% 2,4-dinitrophenol	
S-13	100% S-13	
BPh_3Fe^{2+} + S-13	some S-13	100% BPh_3Fe^{2+} + some S-13
Dicoumarol	30% Dicoumarol	70% Dicoumarol
BPh_3Fe^{2+} + Dicoumarol	100% BPh_3Fe^{2+} + 67% Dicoumarol	33% Dicoumarol
$DPBP_3Fe^{2+}$		100% DPBP
$DPBP_3Fe^{2+}$ + FCCP	100% $DPBP_3Fe^{2+}$ + 100% FCCP	
$DPBP_3Fe^{2+}$ + Dicoumarol	100% $DPBP_3Fe^{2+}$ + 67% Dicoumarol	33% Dicoumarol
oPh_3Fe^{2+}		100% oPh_3Fe^{2+}
oPh_3Fe^{2+} + FCCP	100% FCCP	
BPh-sulfonate$_3Fe^{2+}$		100% BPh-sulfonate$_3Fe^{2+}$
BPh-sulfonate$_3Fe^{2+}$ + FCCP	100% FCCP	
$TMoPh_3Fe^{2+}$		100% $TMoPh_3Fe^{2+}$
$TMoPh_3Fe^{2+}$ + FCCP	100% FCCP	
5-$PhoPh_3Fe^{2+}$		100% 5-$PhoPh_3Fe^{2+}$
5-$PhoPh_3fe^{2+}$ + FCCP	100% FCCP	

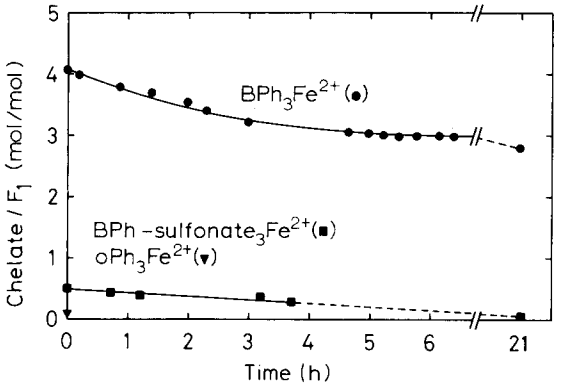


Fig. 8. Binding of BPh_3Fe^{2+} to F_1. 10 μM F_1 was mixed with 250 μM BPh_3Fe^{2+} in a buffer containing 0.25 M sucrose and 10 mM Tris-H_2SO_4 (pH 7.6). 100 μl of this mixture were applied on a 11 X 0.76 cm Sephadex G-25 medium column and eluted with the same buffer at room temperature. For other experimental conditions see text. Concentrations of chelates were determined using an absorption coefficient at 535 nm of 22 $mM^{-1} \cdot cm^{-1}$ for BPh_3Fe^{2+} and BPh-sulfonate$_3Fe^{2+}$ and an absorption coefficient at 510 nm of 11 $mM^{-1} \cdot cm^{-1}$ for oPh_3Fe^{2+}. Molarity of F_1 was calculated assuming a molecular weight of 360 000 and 100% purity.

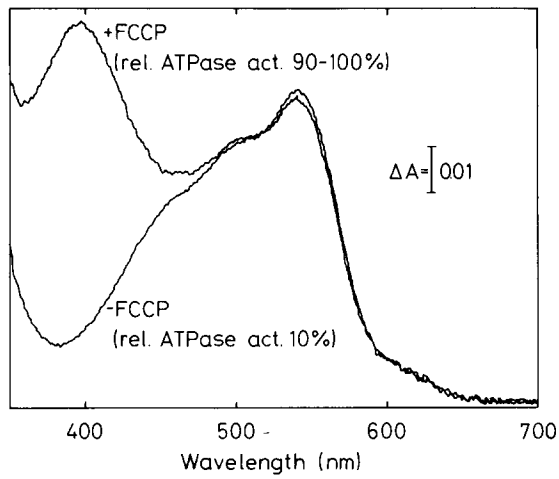


Fig. 9. Spectra of F_1-BPh_3Fe^{2+} and F_1-BPh_3Fe^{2+}-FCCP. The complexes were obtained as described in Fig. 8 and in the text.

Binding of uncouplers to the F_1-BPh_3Fe^{2+} complex

When an uncoupler (2,4-dinitrophenol or FCCP) was included in the enzyme/BPh_3Fe^{2+} mixture before application on the Sephadex column, it did not influence the extent of BPh_3Fe^{2+} binding (cf. Fig. 8) and the presence of uncoupler could be detected spectrophotometrically in the fraction containing the F_1-$(BPh_3Fe^{2+})_3$ complex (Fig. 9). No uncoupler was eluted together with the enzyme in the absence of BPh_3Fe^{2+}. The spectrum of the uncoupler in the eluted F_1-BPh_3Fe^{2+}-uncoupler fraction was shifted to 400 nm, in a manner typical of the interaction of uncouplers with BPh_3Fe^{2+} as found in the absence of F_1 (see Fig. 6). It thus appears that the uncoupler in the eluate in Fig. 9 is bound to the BPh_3Fe^{2+} moiety of the F_1-$(BPh_3Fe^{2+})_3$ complex. The F_1-$(BPh_3Fe^{2+})_3$-uncoupler complex was enzymically active and was similar to F_1 in terms of maximal velocity and K_m for ATP.

When relatively high concentrations of F_1 were titrated with BPh_3Fe^{2+}, complete inhibition could be extrapolated to occur at about 3 mol BPh_3Fe^{2+}/mol F_1 (Fig. 10), in agreement with the binding data.

Effect of bPh_3Fe^{2+} on oligomycin-sensitive ATPase

As reported earlier [5], bPh_3Fe^{2+} also inhibited particulate oligomycin-sensitive mitochondrial ATPase (prepared as 'Complex V' [21]) and this inhibition was similarly relieved by uncouplers. Indeed, as reported elsewhere [22], BPh_3Fe^{2+} appears to be a general inhibitor of enzymes catalyzing the hydrolysis of pyrophosphate bonds.

Effect of BPh_3Fe^{2+} on oxidative phosphorylation

As expected, BPh_3Fe^{2+} also inhibits oxidative phosphorylation, although this effect cannot readily be demonstrated because of the inhibition of electron transport by bathophenanthroline [23,24]. However, as shown in Table IV, addition of BPh_3Fe^{2+} to phosphorylating submitochondrial (Mg^{2+}-ATP) particles from beef heart caused a decrease in ATP synthesis coupled to NADH oxidation, which was greater than the inhibition of NADH oxidase, resulting in a decrease in the P/O ratio. Low concentrations of BPh_3Fe^{2+} stimulated respiration; the reason for this effect is not understood.

Influence of BPh_3Fe^{2+} on the uncoupling effect of FCCP

It has also been possible to show that BPh_3Fe^{2+} is capable of abolishing the uncoupling effect of a typical uncoupler such as FCCP. In the experiment in Fig. 11, the oligomycin-induced respiratory control

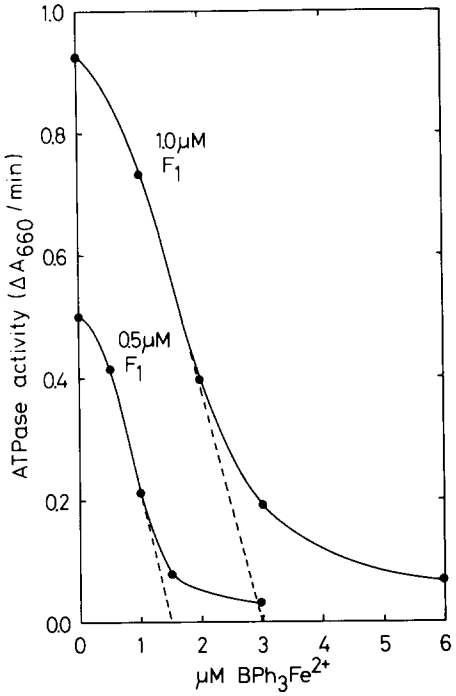


Fig. 10. Inhibition of F_1-ATPase by BPh_3Fe^{2+} at high protein concentrations. ATPase activity was assayed by determining the amount of P_i liberated. The assay medium contained 25 mM Tris-acetate, 30 mM potassium acetate, 3 mM magnesium acetate and 3 mM ATP (pH 7.6). BPh_3Fe^{2+} and F_1 were added at varying concentrations as indicated.

of the NADH oxidase activity of EDTA particles prepared from beef heart mitochondria [25] was measured in the absence and presence of FCCP and with varying concentrations of BPh_3Fe^{2+}. In the absence of FCCP, BPh_3Fe^{2+} did not inhibit respiration, and even caused a moderate increase in oxygen uptake at higher concentrations. In the presence of FCCP, respiration was diminished by low concentrations of BPh_3Fe^{2+} down to the level found in the absence of FCCP. Evidently, BPh_3Fe^{2+} abolished the uncoupling effect of FCCP.

Discussion

The present data confirm and extend previous reports from this laboratory [4–6] concerning the inhibitory effect of certain BPh-metal ion trichelates on mitochondrial F_1-ATPase. The data are consistent with the conclusion that only those metal ions that form stable, octahedral trichelates with bathophenanthroline are effective. There appears to be a high degree of specificity concerning bathophenenthroline and, in particular, its phenyl groups. Omission, replacement, substitution or relocation of these groups greatly diminished or abolished the inhibitory effect. Replacement of the phenanthroline structure by dipyridine, with retention of the phenyl substi-

TABLE IV

EFFECT OF BPh_3Fe^{2+} ON OXIDATIVE PHOSPHORYLATION

Oxidative phosphorylation was measured at 30°C in 2.7 ml reaction mixture containing 170 mM sucrose, 50 mM Tris-acetate (pH 7.6), 5 mM P_i (0.4 μCi $^{32}P/\mu$mol P_i), 2.5 mM ADP, 3 mM magnesium acetate, 1 mM NADH, 20 mM glucose, 50 μg hexokinase and 0.75 mg submitochondrial (Mg-ATP) particles. When approx. 1 μgatom O was consumed, the reaction was quenched by mixing with 0.5 of perchloric acid and the distribution of ^{32}P between isobutanol/benzene and water was determined.

BPh_3Fe^{2+} (μM)	O_2 consumption (μg atom/min per mg)	P/O
0.0	0.57	1.10
5.0	0.74	0.73
10.0	0.91	0.24
15.0	1.02	0.22
18.5	0.38	0.17

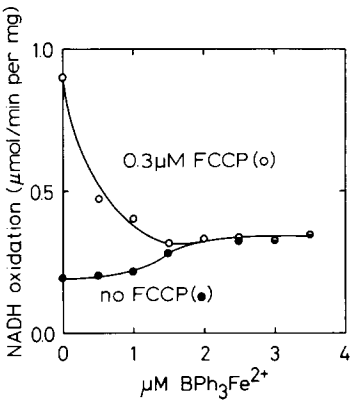


Fig. 11. Influence of BPh_3Fe^{2+} on the uncoupling effect of FCCP. NADH oxidation was measured at 30°C in 3 ml reaction mixture containing 170 mM sucrose, 50 mM Tris-acetate (pH 7.6), 0.2 M NADH, 3 μg oligomycin, 0.1 mg submitochondrial (EDTA) particles and varying concentrations of BPh_3Fe^{2+} in the absence or presence of 0.3 μM FCCP. BPh_3Fe^{2+} and FCCP were added as premixed complex in 30 μl of 50% ethanol.

tuents, preserved the inhibitory effect, although weakening it somewhat.

The reversibility by uncouplers is a unique feature of the inhibition of F_1-ATPase by BPh-metal trichelates, which is not shared by other inhibitors such as aurovertin [16], 4-chloro-7-nitrobenzofuran [17,18], azide [1], or the inhibitor protein of Pullman and Monroy [19]. As has been pointed out [5], this is the first instance where low concentrations of uncouplers are found to act on a vesicle-free, water-soluble, energy-transducing enzyme. This effect is not to be confused with the stimulation of F_1-ATPase by relatively high concentrations of certain phenolates [1] and other anions [26–28] which is observed in the absence of any added inhibitors. From the data reported earlier [5] and in the present paper, it appears that the uncouplers interact directly with the inhibitory chelates, forming stoichiometric adducts, in which the positive charges of the chelates are compensated by the negative charges of the uncouplers.

Data presented earlier [6] and in this paper demonstrate a strong binding between F_1 and the inhibitory chelate, with a stoichiometry of three molecules of chelate per molecule of F_1. As shown in the accompanying paper [7], the binding probably occurs to the β-subunit of F_1. The present data also indicate that the relief of the inhibition by uncouplers most likely involves a binding of the uncouplers to the enzyme-chelate complex, rather than a release of the chelate from the enzyme. The interactions between the enzyme (E), inhibitory chelate (I) and uncoupler (U) may be described by the following equations (for the sake of simplicity written as involving one molecule of each):

$$E + I \rightleftharpoons EI \quad (1)$$

$$EI + U \rightleftharpoons EIU \quad (2)$$

$$I + U \rightleftharpoons IU \quad (3)$$

where E and EIU are active forms of the enzyme, and EI is an inactive form. Direct evidence for the formation of both EI and EIU is presented in Fig. 9. The above equations explain how a substoichiometric amount of an uncoupler (e.g., S-13; cf. Fig. 5) in relation to the amount of BPh_3Fe^{2+} may bring about a virtually complete reactivation of F_1; this will occur if U reacts preferentially with EI (according to Eqn. 2) as compared to its reaction with free I (Eqn. 3). The total amount of uncoupler required for relieving the inhibition will of course be dependent on the amount of free as well as enzyme-bound inhibitor (Fig. 5).

It was concluded earlier [4] that the effect of uncouplers in relieving the inhibition of F_1-ATPase by bPh-metal ion chelates probably is related to their effect on oxidative phosphorylation. This conclusion was based on the arguments that: (a) the effect of uncouplers on oxidative phosphorylation cannot involve F_1, since uncouplers are known to relieve oligomycin-induced respiratory control in F_1-depleted submitochondrial particles [29,30]; and (b) the effect of uncouplers in relieving the inhibition by BPh-metal chelates cannot be due to a direct interaction between inhibitor and uncoupler, because of the substoichiometric amounts of certain uncouplers needed for the relief of inhibition. While the first argument certainly is valid, the second seems to require revision on the basis of the data just presented. Moreover, it appears from the present data that only those chelates that can interact with uncouplers are inhibitors of F_1. Further aspects of the possible mode of action of BPh-metal chelates and uncouplers on F_1-ATP ase are discussed in the accompanying paper [7].

Acknowledgements

This work has been supported by the Swedish Natural Science Research Council and the Swedish Cancer Society.

References

1 Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329
2 Penefsky, H.S. (1979) Adv. Enzymol. 49, 223–280.
3 Ernster, L., Sandri, G., Hundal, T., Carlsson, C. and Nordenbrand, K. (1977) in Structure and Function of Energy Transducing Membranes (Van Dam, K. and Van Gelder, B.S., eds.), BBA Library, vol. 14, pp. 209–222, North-Holland, Amsterdam
4 Phelps, D.C., Nordenbrand, K., Nelson, B.D. and Ernster, L. (1975) Biochem. Biophys. Res. Commun, 63, 1005–1012
5 Phelps, D.C., Nordenbrand, K., Hundal, T., Carlsson, C.,

Nelson, B.D. and Ernster, L. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmiari, F., Slater, E.C. and Siliprandi, N., eds.), pp. 385–400, North-Holland, Amsterdam

6 Carlsson, C., Hundal, T., Nordenbrand, K. and Ernster, L. (1978) in The Proton and Calcium Pumps (Azzone, G.F., Avron, M., Metcalfe, J.C., Quagliariello, E. and Siliprandi, N., eds.), pp. 177–184, North-Holland, Amsterdam
N., eds.), pp. 177–184, North-Holland, Amsterdam

7 Carlsson, C. and Ernster, L. (1981) Biochim. Biophys. Acta 638, 358–364

8 Horstman, L.L. and Racker, E. (1970) J. Biol. Chem. 245, 1336–1344

9 Lee, C.P. and Ernster, L. (1967) Methods Enzymol. 10, 543–548

10 Lindberg, O. and Ernster, L. (1956) Methods Bicohem. Anal. 3, 1–22

11 Lowry, O.H., Rosebrough, N.J. Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275

12 Sillén, L.G. and Martell, A.E. (1964) Stability Constants, The Chemical Society, London

13 Jørgensen, C.K. (1963) Inorganic Complexes, Academic Press, New York.

14 Altman, P.L. and Dittmer Katz, D. (1976) Cell Biology I pp. 207–217, Fed. Am. Soc. Exp. Biol., Bethesda, MD

15 Hanstein, W.G. and Hatefi, Y. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 288–292

16 Lardy, H.A., Connelly, J.L. and Johnson, D. (1964) Biochemistry 3, 1961–1968

17 Ferguson, S.J., Lloyd, W.J., Lyons, M.H. and Radda, G.K. (1975) Eur. J. Biochem. 54, 117–126

18 Ferguson, S.J., Lloyd, W.J. and Radda, G.K. (1975) Eur. J. Biochem. 54, 127–133

19 Pullman, M.E. and Monroy, G.C. (1963) J. Biol. Chem. 238, 3762–3769

20 Pullman, M.E. and Penefsky, H.S. (1963) Methods Enzymol. 6, 277–294

21 Stiggall, D.L., Galante, Y.M. and Hatefi, Y. (1979) Methods Enzymol. 55, 308–315

22 Carlsson, C. and Ernster, L. (1980) in Frontiers of Bioorganic Chemistry and Molecular Biology (Ananchenko, S.N., ed.) pp. 1–9, Pergamon Press, Oxford

23 Tappel, A.L. (1960) Biochim. Pharm. 3, 289–296

24 Phelps, D.C., Harmon, H.J. and Crane, F.L. (1974) Biochem. Biophys. Res. Commun. 59, 1185–1191

25 Lee, C.P. and Ernster, L. (1968) Eur. J. Biochem. 3, 391–400

26 Lambeth, D.O. and Lardy, H.A. (1971) Eur. J. Biochem. 22, 355–363

27 Stockdale, M. and Selwyn, M.J. (1971) Eur. J. Biochem. 21, 416–423

28 Cantey, L.C. and Hammes, G.G. (1973) Biochemistry 12, 4900–4904

29 Ernster, L., Nordenbrand, K., Chude, O. and Juntti, K. (1974) in Membrane Proteins in Transport and Phosphorylation (Azzone, G.F., Avron, M., Metcalfe, J.C., Quagliariello, E. and Siliprandi, N., eds.), pp. 29–41, North-Holland, Amsterdam

30 Lee, C.P., Huang, C.H. and Cierkosz, B.I.T. (1974) in Membrane Proteins in Transport and Phosphorylation (Azzone, G.F., Avron, M., Metcalfe, J.C., Quagliariello, E. and Siliprandi, N., eds.), pp. 161–170 North-Holland, Amsterdam