Bathophenanthroline–ruthenium chelate, a fluorescent inhibitor of F_1-ATPase

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1. INTRODUCTION

We have reported [1–5] that the mitochondrial ATPase F_1 is strongly and specifically inhibited by octahedral metal-trichelates of bathophenanthroline (4,7-diphenyl-1,10-phenanthroline, BPh) and that the inhibition is relieved by uncouplers of oxidative phosphorylation. It was shown that the inhibitor binds to the β-subunit of F_1 and that the reversal of the inhibition by uncouplers involves a binding of the uncouplers to the inhibitory chelates, resulting in a catalytically active enzyme–inhibitor–uncoupler complex. Evidence was also obtained which indicated that this inhibition may occur generally with enzymes catalyzing the reversible hydrolysis of pyrophosphate bonds, and it was speculated that the inhibitory chelates may act by blocking a proton transfer between the active centers of these enzymes and the surrounding medium which may be essential for the catalysis [6].

The above studies were performed mainly with the Fe^{2+} trichelate of BPh (BPh_3Fe^{2+}), although it was shown that BPh_3Ni^{2+} and BPh_3Ru^{2+} are equally efficient in bringing about an uncoupler-reversible inhibition of F_1-ATPase. Recently, we have extended these studies by using BPh_3Ru^{2+} [7], taking advantage of the fluorescence of this chelate. This paper is a brief report on the interaction of BPh_3Ru^{2+} with soluble F_1.

Abbreviations: BPh, bathophenanthroline (4,7-diphenyl-1,10-phenanthroline); DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 1799, 2,6-dihydroxy-1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptan-4-one; PEP, phosphoenolpyruvate; PK, pyruvate kinase; TCA, trichloroacetic acid; BSA, bovine serum albumin.

2. MATERIALS AND METHODS

Mitochondrial F_1-ATPase was purified from beef heart mitochondria according to [8]. It had a specific activity of approximately 100 μmol · min^{-1} · mg protein^{-1}. The enzyme was stored at 4°C in a 50% saturated $(NH_4)_2SO_4$ suspension containing 0.125 M sucrose, 5 mM Tris–SO_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–SO_4 and 2 mM EDTA (pH 7.6). ATPase activity was assayed at 30°C in a 1 ml reaction mixture containing 25 mM Tris–Ac, 30 mM KAc, 3 mM MgAc, 4 mM ATP, 15 mM PEP, 50 μg PK, 18 μg F_1 and varying concentrations of BPh_3Ru^{2+} and/or FCCP. The reaction was stopped after 1 min by addition of 50 μl 100% TCA and the amount of P_i liberated was determined by the method of [9]. Molarity of F_1 is given assuming a molecular weight of 360 000 and 100% purity.

ESU particles were prepared from beef heart mitochondria by treatment of 'EDTA particles' [10] with Sephadex and urea as in [11].

Protein was determined by the method of Lowry et al. [12], using BSA as standard.

BPh_3Ru^{2+} was obtained as a kind gift from Dr David Sigman, Department of Biological Chemistry, University of California, Los Angeles, USA. Fluorescence measurements were made at 20°C

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with an Aminco-Bowman spectrophotometer, with 470 nm as excitation wavelength and 600 nm as emission wavelength, in a medium containing 50 mM Tris–Ac (pH 7.5).

3. RESULTS AND DISCUSSION

The excitation spectrum of BPh_3Ru^{2+} in 50 mM Tris–Ac (pH 7.5), reveals two excitation maxima, at 440 and 470 nm respectively (fig.1A). These maxima are close to those in the visible adsorption spectrum, viz. 430 and 460 nm. The emission spectrum shows a maximum at 600 nm, when 470 is used as excitation wavelength (fig.1B). Addition of F_1 enhances the BPh_3Ru^{2+} fluorescence and this fluorescence enhancement is abolished by the uncoupler FCCP (fig.2). The effect of FCCP is duplicated by other uncouplers including 1799 and DNP. Also, in accordance with earlier findings showing a direct interaction between inhibitory bathophenanthroline chelates and uncouplers, FCCP abolishes the BPh_3Ru^{2+} fluorescence in the absence of F_1.

Titrations with F_1 at a constant chelate concentration shows a hyperbolic relationship between the extent of fluorescence enhancement and the F_1 concentration (fig.3A), with an extrapolated enhancement of 5.4-fold at infinite F_1 concentration (fig.3B). In fig.4 it can be seen that the fluorescence-enhancement of BPh_3Ru^{2+} is parallel to the inhibition of the ATPase activity, and that the fluorescence-enhancement is abolished by FCCP along with the reversal of the inhibition. The F_1-induced BPh_3Ru^{2+} fluorescence is virtually independent of the pH of the medium in the range of 5–9. As expected, the fluorescence-enhancement, decreases when the temperature of the medium is increased.

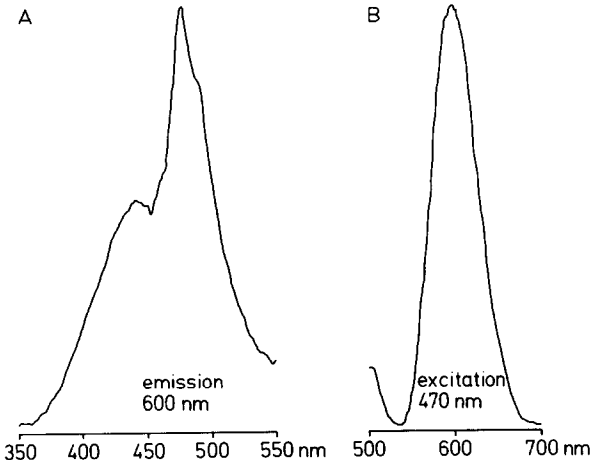

Fig.1. Fluorescence excitation spectra (A) and emission spectra (B) of BPh_3Ru^{2+}.

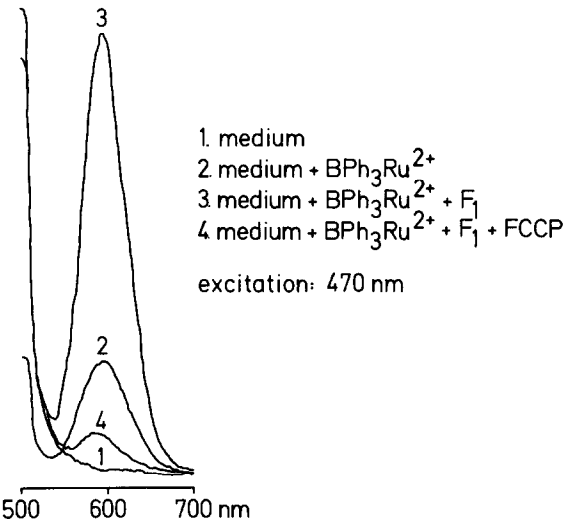

Fig.2. Effect of F_1, in the absence and presence of FCCP, on the fluorescence of BPh_3Ru^{2+}.

ATP, ADP or ADP-Mg, at a concentration of 4 mM, does not effect the enhancement of BPh_3Ru^{2+} fluorescence by F_1. Nor does the ATPase inhibitor protein used at concentrations totally inhibiting the ATPase activity, influence the F_1-induced BPh_3Ru^{2+} fluorescence-enhancement.

Figure 5 shows that cold-inactivation of F_1 results in an increase of the fluorescence-enhancement of BPh_3Ru^{2+} by F_1, probably due to the fact that F_1 dissociates in the cold and more binding sites of the enzyme become exposed to the chelate. It can also be seen in fig.5 that inhibition of F_1 by photooxidation in the presence of Rose Bengal [13], results in a decrease of the maximal BPh_3Ru^{2+} fluorescence-enhancement by F_1.

Rebinding of F_1 to ESU particles causes a

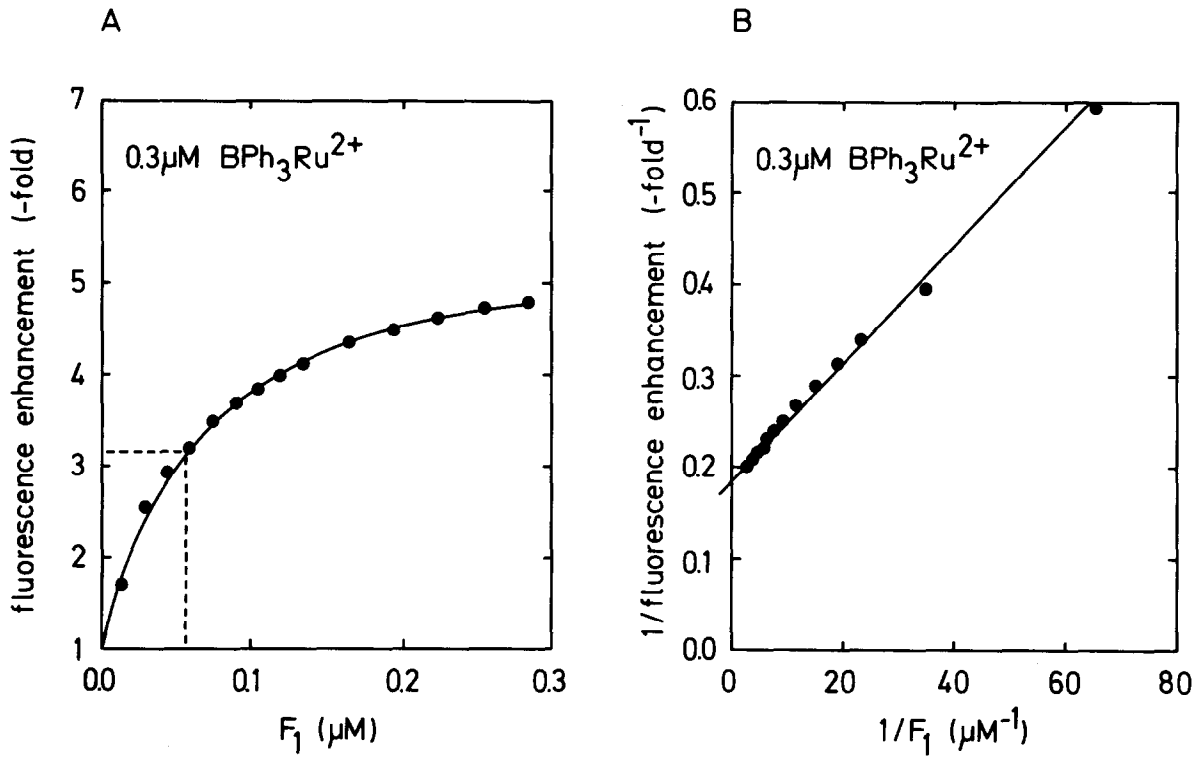

Fig.3. Relationship between the extent of BPh_3Ru^{2+} fluorescence-enhancement and F_1 concentration.

quenching of the F_1-induced BPh_3Ru^{2+} fluorescence-enhancement, suggesting that the hydrophobic chelate binds unspecificially to the membrane. The effect is consistent with earlier results [2] showing that membrane-bound F_1, compared to the soluble enzyme, needs much higher chelate concentrations for maximal inhibition.

The F_1-induced enhancement of BPh_3Ru^{2+} fluorescence proved not to be specific, other proteins, e.g., BSA, insulin and yeast PP_iase – showing the same effect. BSA is just as effective as F_1 in enhancing the BPh_3Ru^{2+} fluorescence, while yeast PP_iase is slightly more effective than F_1 and insulin slightly less. Also in those cases uncouplers, such as FCCP, abolish the fluorescence enhancement.

Scatchard plot representation of titrations with BPh_3Ru^{2+} at constant F_1 concentrations shows a non-linear relationship (fig.6), probably due to the strong association between BPh_3Ru^{2+} and F_1 and some unspecific binding of the chelate to the enzyme at high chelate concentrations. Not taking into account the points at the lowest and the highest chelate concentrations, each mol of F_1 could be extrapolated to bind approximately 3 mol of BPh_3Ru^{2+}.

Titrations with F_1 at constant BPh_3Ru^{2+} concentrations show that half-maximal fluorescence enhancement is reached at a molar ratio of BPh_3Ru^{2+}:F_1 of 1.5 (fig.3A) indicating that F_1 at maximal fluorescence enhancement binds 3 mol BPh_3Fe^{2+}/mol F_1.

These data are in good agreement with the results [4] obtained with the Fe^{2+}-chelate, showing the formation of an enzymically inactive complex between F_1 and BPh_3Ru^{2+}, containing 3 mol BPh_3Fe^{2+}/mol F_1.

It thus appears that BPh_3Ru^{2+} binds with high affinity to the same site of F_1 as earlier concluded for BPh_3Fe^{2+} [5], i.e., to the catalytic center of the

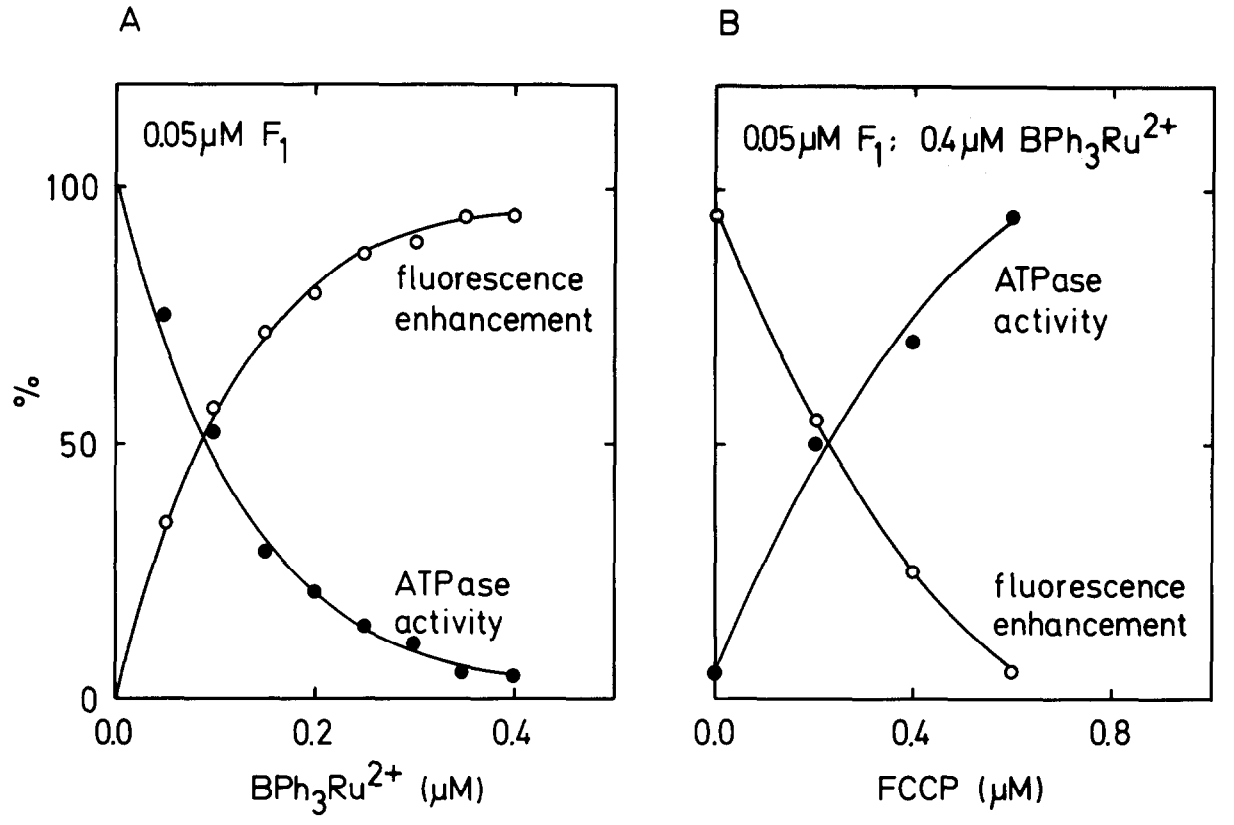

Fig.4. Correlation between the enhancement of BPh_3Ru^{2+} fluorescence by F_1 and the inhibition and reactivation of ATPase activity by BPh_3Ru^{2+} and FCCP, respectively.

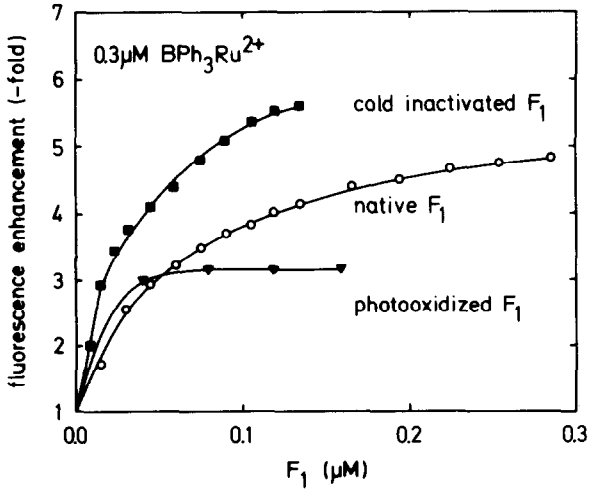

Fig.5. Effects of cold-inactivated F_1 and of F_1 photooxidized in the presence of Rose Bengal on the fluorescence of BPh_3Ru^{2+}. Cold-inactivation: F_1 at a protein concentration of 4 mg/ml was incubated for 6 h at 0°C (~1% activity left). Photooxidation: F_1 at a protein concentration of 2 mg/ml, was incubated for 0.5 h in the presence of 10 μM Rose Bengal, in a small test tube immersed in a large volume of water kept at room temperature. The sample was illuminated with a 250 W slide projector during incubation (~1% activity left).

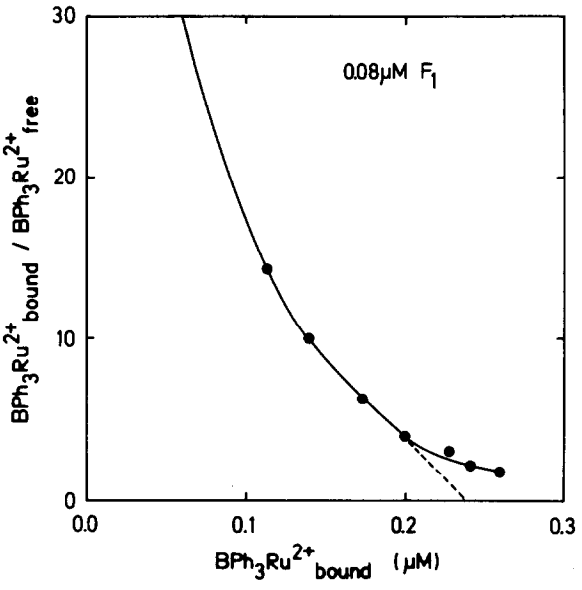

Fig.6. Scatchard plot representation of titration with BPh_3Ru^{2+} at constant F_1 concentration.

enzyme located on the β-subunit. Attempts to provide direct evidence for this conclusion, using the isolated β-subunit, are in progress. By virtue of its fluorescence, BPh_3Ru^{2+} may seem as a valuable probe for F_1 and its catalytic subunit.

ACKNOWLEDGEMENT

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