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Possible involvement of the 29 kDa protein in H^+-ATPase in the action of cationic uncoupler of oxidative phosphorylation. Effect of the (*o*-phenanthroline)$_2$-Cu^{2+} complex as a cationic uncoupler

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The divalent cation (*o*-phenanthroline)$_2$-Cu^{2+} complex was found to uncouple oxidative phosphorylation in mitochondria. Its uncoupling activity depended on inorganic phosphate (P_i) in the incubation medium, and was inhibited by the SH-reagent *N*-ethylmaleimide, and retarded by ATP. The uncoupling by the (*o*-phenanthroline)$_2$-Cu^{2+} complex was suggested to be due to its modification of sulfhydryl groups in the 29 kDa protein in H^+-ATPase.

Uncoupling of oxidative phosphorylation in mitochondria by hydrophobic cations such as DDA^+ and TBA^+ is generally thought to be due to dissipation of the membrane potential caused by electrophoretic transfer of these cations through the mitochondrial inner membrane [1,2]. However, from results on the effects of the divalent cationic cyanine dyes tri-S-C_4(5) and tri-S-C_7(5) on the functions of rat liver mitochondria [3–5], and of noise analysis of bilayer membranes treated with these dyes [6], we have concluded that the uncoupling by these dyes is due to their direct actions in the mitochondrial membrane protein, rather than their transfer into the mitochondria.

The (*o*-phenanthroline)$_2$-Cu^{2+} complex has been shown to induce the oxidation of SH groups in proteins in the presence of oxygen [7], and recently it was found to cause specific intermolecular cross-linkage of the 29 kDalton protein in highly purified H^+-ATPase [8]. Since the (*o*-phenanthroline)$_2$-Cu^{2+} complex is a hydrophobic divalent cation, it seemed interesting to examine whether it acts as a cationic uncoupler like cyanine dyes.

The (*o*-phenanthroline)$_2$-Cu^{2+} complex was prepared by the method of Kobashi [9]. The other reagents used were commercial products.

Mitochondria were isolated from the liver of adult male Wistar rats as reported by Myers and Slater [10]. Mitochondrial respiration was monitored with a Clark oxygen electrode at 25°C. Two types of incubation medium with the following compositions were used to examine the effect of P_i: $+P_i$ medium consisted of 200 mM sucrose/2 mM $MgCl_2$/1 mM Na_2EDTA/10 mM potassium phosphate buffer (pH 7.4); $-P_i$ medium had the same composition as $+P_i$ medium, but with 10

Abbreviations: P_i, inorganic phosphate; tri-S-C_4(5), 2,2′-[3-[2-(3-butyl-4-methyl-2-thiazolin-2-ylidene)ethylidene]propenylene]-bis[3-butyl-4-methylthiazolinium iodide]; tri-S-C_7(5), 2,2′-[3-[2-(3-heptyl-4-methyl-2-thiazolin-2-ylidene)ethylidene]propenylene]bis[3-heptyl-4-methylthiazolinium iodide], also named NK-19 or Platonin; DDA^+, *N*,*N*-dibenzyl-*N*,*N*-dimethylammonium; TBA^+, tetrabutylammonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malonitrile.

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mM Tris-HCl buffer instead of potassium phosphate buffer.

The $[^{32}P]P_i$–ATP exchange reaction was determined by the method of Pullman [11]. The reaction mixture consisted of 50 mM sucrose/20 mM KCl/0.1 mM Na_2EDTA/3 mM $MgCl_2$/5 mM Tris-HCl buffer (pH 7.4)/3 mM potassium phosphate buffer, containing 1 μCi $[^{32}P]P_i$ (pH 7.4). A known amount of (*o*-phenanthroline)$_2$-Cu^{2+} complex was added to the mitochondrial suspension (2.0 mg protein/ml) containing 1 mM ATP at 25°C, and after 2 min the amount of $[^{32}P]$ATP in the medium was determined in an Aloka liquid scintillation spectrometer LSC-602.

SDS-polyacrylamide gel electrophoresis was performed by the method of Fairbanks et al. [12] in the absence of 2-mercaptoethanol. Proteins were stained by Coomassie brilliant blue R-250 and subjected to densitometry in a Shimadzu dual-wavelength TLC scanner CS-910.

As shown in Fig. 1A, 60 μM (*o*-phenanthroline)$_2$-Cu^{2+} complex gradually accelerated the respiration of state 4 mitochondria suspended in $+P_i$ medium with succinate (plus 1 μg/mg rotenone) as substrate. About 3 min after addition of the (*o*-phenanthroline)$_2$-Cu^{2+} complex, the respiration rate attained a maximum of more than 4-times that in state 4. The (*o*-phenanthroline)$_2$-Cu^{2+} complex also released oligomycin-inhibited state 3 respiration after a lag phase (Fig. 1B). Results of the effect of the (*o*-phenanthroline)$_2$-Cu^{2+} complex on the $[^{32}P]P_i$–ATP exchange reaction of mitochondria 2 min after its addition are summarized in Table I. At 95 nmol/mg protein it inhibited half the $[^{32}P]P_i$–ATP exchange reaction of mitochondria. Thus, the (*o*-phenanthroline)$_2$-Cu^{2+} complex is concluded to be an uncoupler of oxidative phosphorylation. This uncoupling dependent on P_i: when mitochondria were incubated in $-P_i$ medium, no release of state 4 respiration by (*o*-phenanthroline)$_2$-Cu^{2+} complex was observed (Fig. 1A), as with the cyanine dyes tri-S-$C_4(5)$ and tri-S-$C_7(5)$ [3–5].

TABLE I

EFFECT OF THE (*o*-PHENANTHROLINE)$_2$-Cu^{2+} COMPLEX ON THE $[^{32}P]P_i$–ATP EXCHANGE REACTION

Concentration of the (*o*-phenanthroline)$_2$-Cu^{2+} complex (nmol·mg^{-1})	$[^{32}P]P_i$–ATP exchange [a] (% inhibition)
0	0
95	49.5 ± 7.5
190	87.1 ± 0.7

* The rate of $[^{32}P]P_i$–ATP exchange in the absence of the (*o*-phenanthroline)$_2$-Cu^{2+} complex was 311 nmol P_i/mg protein per min. Values listed are means for at least three determinations.

The SH reagent *N*-ethylmaleimide inhibited the uncoupling by the (*o*-phenanthroline)$_2$-Cu^{2+} complex. As shown in Fig. 2A, when state 4

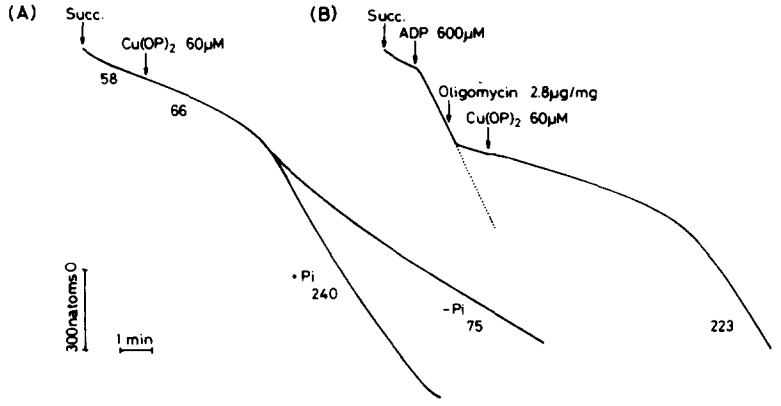

Fig. 1. Effect of the (*o*-phenanthroline)$_2$-Cu^{2+} complex on state 4 respiration in the presence and absence of P_i (A), and on the oligomycin-inhibited state 3 respiration of mitochondria (B). Mitochondria (0.7 mg protein/ml) were incubated in either $+P_i$ or $-P_i$ medium in a total volume of 2.53 ml. Numbers adjacent to traces are respiratory rates in natom O/min. Substrate: 10 mM succinate with 1 μg rotenone/mg protein.

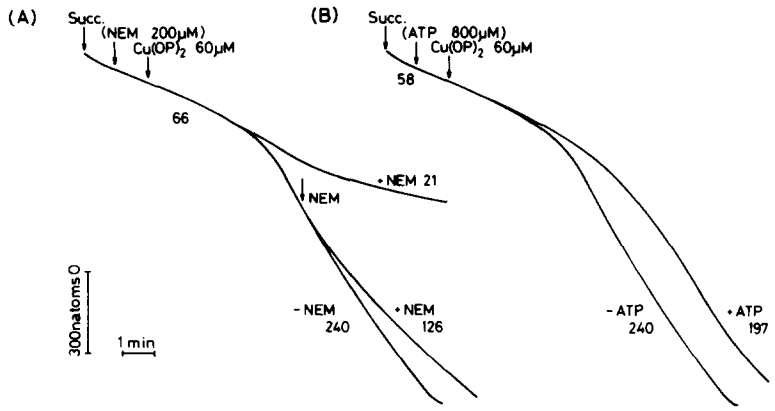

Fig. 2. Effects of *N*-ethylmaleimide (A) and ATP (B) on uncoupling by the (*o*-phenanthroline)$_2$-Cu^{2+} complex. Mitochondria were incubated in $+P_i$ medium. Experimental conditions were as for Fig. 1.

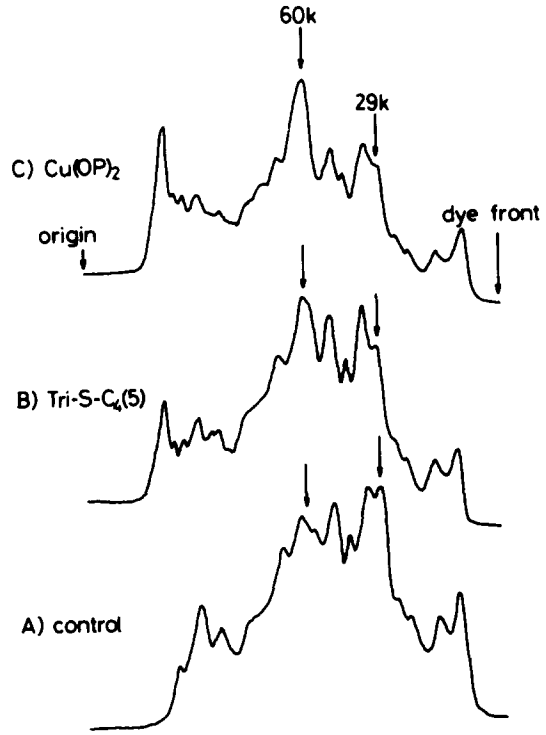

Fig. 3. Effects of tri-S-C_4(5) and the (*o*-phenanthroline)$_2$-Cu^{2+} complex on the 29 kDa protein in mitochondrial membrane. Mitochondria were incubated in $+P_i$ medium either in the absence of cationic uncoupler (A), or in the presence of 200 μM tri-S-C_4(5) (B) or 60 μM (*o*-phenanthroline)$_2$-Cu^{2+} complex (C). Then the mitochondria were solubilized with 0.1% SDS, and subjected to SDS-polyacrylamide gel electrophoresis on 5.6% acrylamide in 40 mM Tris buffer at pH 7.4. After electrophoresis, the amounts of proteins were measured by densitometry of the gel stained with Coomassie brilliant blue R-250 were determined by densitometry.

mitochondria were preincubated with *N*-ethylmaleimide, no release of respiration by the (*o*-phenanthroline)$_2$-Cu^{2+} complex was observed: uncoupling was completely inhibited. *N*-Ethylmaleimide also inhibited the uncoupling when added to mitochondria uncoupled by the (*o*-phenanthroline)$_2$-Cu^{2+} complex, though less than when it was added before the (*o*-phenanthroline)$_2$-Cu^{2+} complex. Furthermore, ATP was found to have a protective effect against uncoupling by the (*o*-phenanthroline)$_2$-Cu^{2+} complex. As shown in Fig. 2B, ATP retarded the release of respiration of state 4 mitochondria in $+P_i$ medium. However, it had no effect on uncoupled mitochondria.

Recently, Joshi and Torok reported that the (*o*-phenanthroline)$_2$-Cu^{2+} complex caused intermolecular cross-linkage of the 29 kDa protein in H^+-ATPase from bovine heart mitochondria [8]. We also observed that the amount of 29 kDa protein from rat liver mitochondria treated with 60 μM (*o*-phenanthroline)$_2$-Cu^{2+} complex or with 200 μM tri-S-C_4(5) decreased with the appearance of proteins of about 60 kDa on SDS-polyacrylamide gel electrophoresis (Fig. 3). On the other hand, in the presence of *N*-ethylmaleimide, these two uncouplers failed to cause cross-linkage of 29 kDa protein, probably due to alkylation of the sulfhydryl group in the 29 kDa protein by *N*-ethylmaleimide.

Discussion

In this study we found that the divalent cation (o-phenanthroline)$_2$-Cu^{2+} complex is an uncoupler of oxidative phosphorylation in mitochondria. This uncoupling differed from those by weakly acidic uncouplers such as FCCP and SF 6847 [13] as follows: (i) It required P_i; (ii) it was inhibited by N-ethylmaleimide; (iii) it was retarded by ATP; and (iv) it involved modification of the 29 kDa protein in H^+-ATPase. Features (i) to (iii) have been observed in uncoupling by the cyanine dyes tri-S-C_7(5) and tri-S-C_4(5) [3–5], and in this study we found that these dyes also caused cross-linkage of the 29 kDa protein. Thus these features are apparently common to the uncouplings by hydrophobic cations.

ATP was reported to have a protective effect against the induction of S–S cross-linkages of proteins by the (o-phenanthroline)$_2$-Cu^{2+} [7,8]. The protective effect of ATP against uncoupling by the (o-phenanthroline)$_2$-Cu^{2+} complex and cyanine dyes was weaker than that by SH-reagents such as N-ethylmaleimide, suggesting that it was due to non-covalent shielding of SH groups.

The role of P_i in induction of uncoupling could be due to a nonspecific action on the surface of the membrane, making the membrane accessible to the (o-phenanthroline)$_2$-Cu^{2+} complex, since P_i reduces the membrane surface tension [6], and cyanine dyes increase phospholipid bilayer conductance in the presence, but not the absence of P_i [14].

From studies on the effects of cyanine dyes on oxidative phosphorylation in mitochondria [3–5], and on the phospholipid bilayer membrane [6], we concluded that these cationic uncouplers dissipate the membrane potential by acting on the membrane, but not by their electrophoretic transfer across the membrane. Furthermore, as shown in this study, the actions by the (o-phenanthroline)$_2$-Cu^{2+} complex and cyanine dyes can be regarded as essentially the same. Thus, the 29 kDa protein in the mitochondrial membrane could be directly related to the uncoupling actions of both the (o-phenanthroline)$_2$-Cu^{2+} complex and cyanine dyes: modification of the state or conformation of this protein, including its cross-linkage, presumably leads to uncoupling.

Ca^{2+} is also known to uncouple oxidative phosphorylation in a quite similar manner to these uncouplers [15]: it requires P_i for uncoupling and its uncoupling effect is inhibited by N-ethylmaleimide or ATP. The uncoupling by Ca^{2+} is thought to be caused by its transfer into the matrix space of mitochondria in concert with the penetration of P_i. However, Moreno-Sánchez recently reported that Ca^{2+} regulates oxidative phosphorylation by acting on the H^+-ATPase and the adenine nucleotide translocator [16,17]. This suggests that the uncoupling action of Ca^{2+} is due not only to its penetration into mitochondria, but also to its direct action on mitochondrial membrane proteins. In preliminary experiments, we found that Ca^{2+} prevented the induction of uncoupling and also cross-linkage of 29 kDa protein by tri-S-C_4(5). These results suggest that Ca^{2+} interacts with 29 kDa protein, though it does not induce cross-linkage, and that modification of the state of 29 kDa protein is important for uncoupling by Ca^{2+} as well as by cyanine dyes and (o-phenanthroline)$_2$-Cu^{2+}.

The 29 kDa protein is reported to be a component of the H^+-ATPase [8,18], and SH groups in this protein are important for regulation of oxidative phosphorylation [18–20]. This protein is also supposed to be an adenine nucleotide translocator [8]. However, its role in ATP synthesis is unknown. Studies on the interaction between this protein and cationic uncouplers should be helpful in understanding the exact mechanism of oxidative phosphorylation as well as the mechanism of uncoupling.

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