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A SPECIFIC UNCOUPLER-BINDING PROTEIN IN *TETRAHYMENA PYRIFORMIS* AND *PARACOCCUS DENITRIFICANS*

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Summary

The uncoupler of mitochondrial oxidative phosphorylation, 2-nitro-4-azido-carbonylcyanide phenylhydrazone (N_3CCP) which is capable of photoaffinity labeling has been used to examine the effect of uncouplers on the energy conserving membranes of *Paracoccus denitrificans* and *Tetrahymena pyriformis*. The N_3CCP uncouples respiration in *P. denitrificans* and *T. pyriformis* cells with $U_{1/2}$ values of 1.05 μM and 0.24 μM, respectively. Binding studies show the presence of 0.65 ± 0.05 high affinity sites per cytochrome *a* with a K_d of 0.5 ± 0.1 μM in *P. denitrificans* membranes and 1.4 ± 0.2 sites per cytochrome a_2 with a K_d of 0.4 ± 0.1 μM in *T. pyriformis* membranes. Irradiation of [^{3}H]-N_3CCP bound to the membranes leads to a covalent linking of the radioactive uncoupler to a peptide of 10—15 kdaltons as analyzed by SDS-polyacrylamide gel electrophoresis. It is concluded that these two microbial systems contain a specific high affinity uncoupler binding site very similar to that of mammalian mitochondria (Katre, N.V. and Wilson, D.F. (1978) Arch. Biochem. Biophys. 191, 647—656).

Introduction

In our earlier papers [1,2] the uncoupler 2-nitro-4-azido-carbonylcyanide phenylhydrazone (N_3CCP), which was capable of photoaffinity labeling, was

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Abbreviations: N_3CCP, 2-nitro-4-azido-carbonylcyanide phenylhydrazone; Mops, 3-(*N*-morpholino)-propanesulfonic acid.

used to determine the nature of interaction between uncouplers and the mitochondrial membrane. It was found that this uncoupler bound to specific high affinity binding sites which involved a proteolipid(s) of 10—15 kdaltons on SDS-polyacrylamide gel electrophoresis. This proteolipid uncoupler binding site was present at a stoichiometry of approx. 1.2 sites per cytochrome *a*. Uncoupler binding to this site was of functional importance, the dissociation constant K_d correlated with the uncoupling activity of N_3CCP ($U_{1/2}$ = 0.2 μM). Other uncouplers competed with N_3CCP for the high affinity uncoupler binding site, their K_i values correlating with their uncoupling activities. After photolysis, the N_3CCP treated mitochondria remained uncoupled and could not, in contrast to samples without photolysis, be recoupled by adding bovine serum albumin.

Having established that uncouplers of oxidative phosphorylation act at a specific binding site, it is important to know whether such a binding site also exist in energy conserving membranes of lower species. This paper describes the interaction of the photoaffinity labeled uncoupler N_3CCP with membrane vesicles from the prokaryotic organism *Paracoccus denitrificans* and with mitochondria from the protozoan *Tetrahymena pyriformis*. It was found that the uncoupler N_3CCP was bound specifically to high affinity binding sites on both the energy conserving membranes. This site was a peptide of molecular weight 10 000—15 000 by SDS-polyacrylamide gel electrophoresis. The uncoupler bound to these sites with dissociation constants (K_d) correlating with their uncoupling activities. The stoichiometry of the high affinity binding sites was 0.65 per cytochrome *a* in *P. denitrificans* and 1.4 per cytochrome a_2 in *T. pyriformis*.

Materials and Methods

N_3CCP synthesized as described in the earlier papers [1,2] was used. The tritiated N_3CCP [2] had a specific activity of 50 mCi/mmol. *Paracoccus denitrificans* was grown aerobically at 30°C as described previously [3] and membrane fragments containing the respiratory chain components were prepared by the method of Scholes and Smith [4]. *Tetrahymena pyriformis* were grown and harvested as described by Kilpatrick and Erecinska [5] and mitochondria were isolated in 0.25 M sucrose, 10 mM KCl, 5 mM Mops and 0.2 mM EDTA, pH 6.2 medium by the method given in detail in the same reference [5].

The cytochrome content was measured by the difference in absorption between the oxidized and reduced forms of the cytochromes. The millimolar extinction coefficients used for the calculation were 24.0 for 615 nm minus 640 nm for cytochrome a_2 [5] of *T. pyriformis* mitochondria and 26.4 for 605 nm minus 630 nm for cytochrome *a* of *P. denitrificans* [6].

Binding of radioactive N_3CCP to the membranes was carried out as described in earlier papers [1,2]. Uncoupling activity of the N_3CCP was measured in suspensions of whole cells of both *P. denitrificans* and *T. pyriformis*. The substrate used was glucose (10 mM) for *P. denitrificans* and malate (5 mM) plus glutamate (5 mM) for *T. pyriformis* mitochondria. The respiratory rate as measured with an oxygen electrode was plotted against uncoupler concentration in order to obtain the uncoupling activity. Covalent labeling of the membrane sus-

pensions with radioactive N_3CCP and the separation of the peptides by SDS-polyacrylamide gel electrophoresis were carried out as described earlier [2], by method of Weber and Osborn [7].

Results

The concentration of N_3CCP required to cause half-maximal stimulation of respiration ($U_{1/2}$) was 0.24 μM in *P. denitrificans* cells and 1.05 μM in *T. pyriformis* cells. The uncoupling activity of N_3CCP for mitochondria isolated from *T. pyriformis* could not be measured because the respiratory control of these mitochondria was very low, about 1—2, and hence the stimulation with uncouplers could not be accurately determined.

Binding studies

The distribution of N_3CCP obtained from the equilibrium binding studies to the membrane suspensions was analysed as Scatchard plots. In the case of the *P. denitrificans* the absorbance and the radioactivity measurements gave good agreement. These results showed the presence of high affinity as well as low affinity binding sites for N_3CCP on the membrane of the prokaryote. The radioactivity measurements of equilibrium binding of N_3CCP in the dark (Fig. 1) indicated that there were 0.65 ± 0.05 high affinity sites per cytochrome *a* on the membrane and N_3CCP bound to these sites had a dissociation

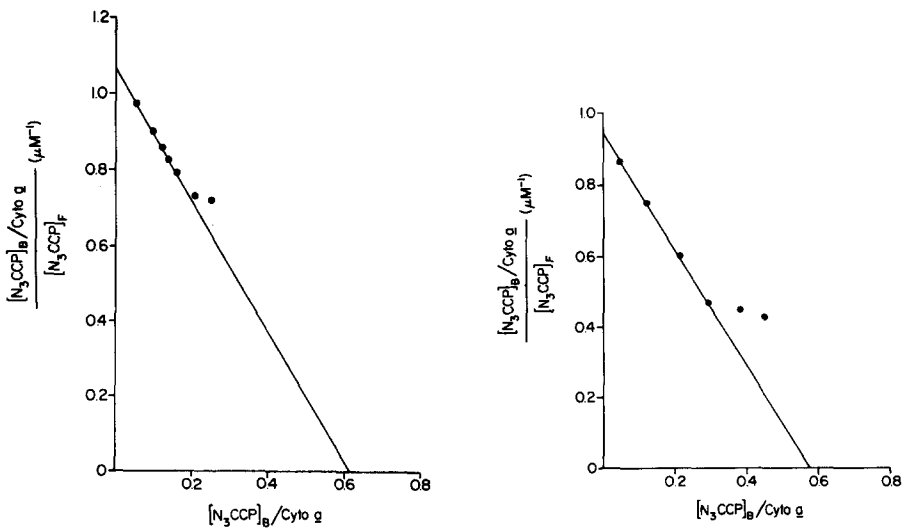

Fig. 1. Scatchard plot of binding of radioactive N_3CCP to *P. denitrificans*. *P. denitrificans* cells were suspended at 2.6 μM cytochrome *a* in a medium containing sucrose (0.25 M) and phosphate buffer (0.01 M), pH 7.4. The total concentrations of radioactive N_3CCP added ranged from 0.2 μM to 3 μM. Radioactivity was measured in pellets and supernatants after centrifugation, and the respective N_3CCP concentration was calculated.

Fig. 2. Scatchard plot of binding of nonradioactive N_3CCP to *P. denitrificans*. *P. denitrificans* cells were suspended at 1.05 μM cytochrome *a* in a medium containing sucrose (0.25 M) and phosphate buffer (0.01 M) pH 7.4. The total concentrations of N_3CCP added ranged from 0.3 μM to 2 μM. The concentration of free N_3CCP in the supernatant was measured by absorbance at 385 nm after centrifugation. The molar extinction coefficient for N_3CCP is 2.6×10^4 at 385 nm.

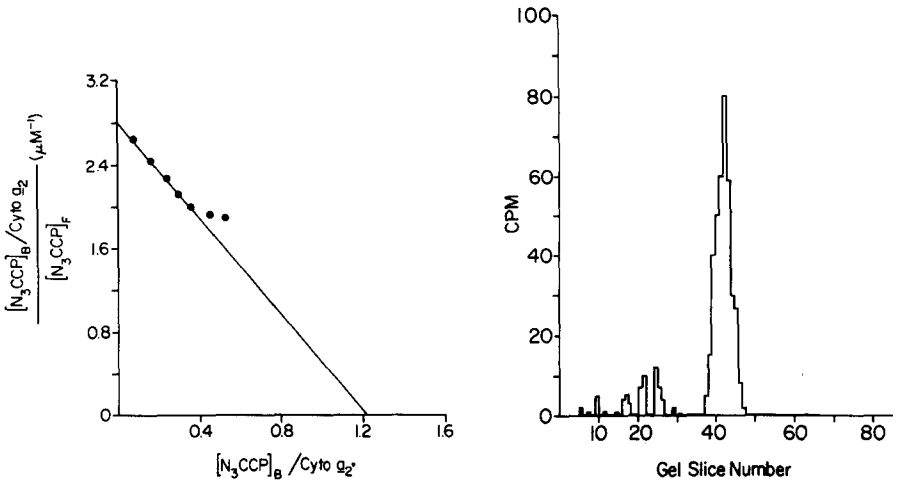

Fig. 3. Scatchard plot of binding of radioactive N_3CCP to mitochondria from *T. pyriformis*. These mitochondria were suspended to 3.8 μM cytochrome a_2 in a medium containing sucrose (0.25 M), Mops (5 mM) and EDTA (0.2 mM), pH 7.2. The total concentrations of N_3CCP added ranged from 0.6 μM to 4.6 μM. Radioactivity was measured in pellets and supernatants after centrifugation and the respective N_3CCP concentrations was calculated.

Fig. 4. Polyacrylamide-SDS gel electrophoresis of *P. denitrificans* membranes photolysed with radioactive N_3CCP (1.5 nmol/mg protein). The membranes were digested with 2—4% SDS, containing 10% glycerol, 0.01 M sodium phosphate pH 7, in presence of mercaptoethanol 9% acrylamide gels were loaded with 200—250 μg protein. The gels were sliced and the radioactivity in each slice measured.

constant (K_d) of 0.5 ± 0.10 μM. Measurement of the uncoupler in the supernatant fraction by its absorbance at 385 nm gave rise to similar Scatchard plots (Fig. 2) and showed the presence of 0.6 ± 0.10 high affinity binding sites for N_3CCP per cytochrome *a* and the K_d value was 0.6 ± 0.03 μM.

In the equilibrium binding studies with mitochondria from *T. pyriformis*, the measurements using radioactive N_3CCP showed the presence of high affinity uncoupler binding sites at a concentration of 1.4 ± 0.2 sites per cytochrome a_2 (Fig. 3). The dissociation constant (K_d) for N_3CCP bound to these sites was 0.4 ± 0.1 μM. In this case, the binding studies could not be carried out by measuring the absorbance of the N_3CCP remaining in the supernatant fraction because the supernatants obtained by centrifugation were turbid and this interfered with spectral measurements of N_3CCP.

Covalent insertion of N_3CCP in the membranes

The membrane fragment suspension from *P. denitrificans* irradiated in the presence of 0.5 nmol radioactive N_3CCP per mg protein, resulted in 50% of the uncoupler being covalently bound to the membrane. Spheroplasts treated similarly were found to be uncoupled, and treatment with bovine serum albumin did not restore respiratory control, in contrast to control spheroplasts treated without irradiation or with irradiation in absence of N_3CCP. In general the results obtained were similar to those in rat liver, beef heart, pigeon heart, and pigeon breast mitochondria [1,2].

Identification of the high affinity uncoupler binding site

Polyacrylamide-SDS gel electrophoresis of the covalently labeled membranes from *P. denitrificans* showed that 80% of the total radioactivity was incorporated into a single peak, with an apparent molecular weight of 14 000 (Fig. 4). The densitometer traces of the 600 nm absorbance of the stained gels showed the presence of a peptide band which corresponded in position to the radioactive peak. Similar results were obtained for covalently labeled mitochondria of *T. pyriformis*. In this case the apparent molecular weight of the labeled peptide was 12 000—13 000. In control experiments, in which the *P. denitrificans* particles or *T. pyriformis* mitochondria were irradiated in the absence of N_3CCP, or tritiated N_3CCP added in the dark without irradiation resulted in no peak of radioactivity in the gels.

Methods for obtaining pure electron transfer complexes or ATPase complex from *T. pyriformis* mitochondria or from membranes of *P. denitrificans* have not yet been developed. The fractionation methods with detergents and salts used for obtaining these complexes from pigeon or beef heart mitochondria proved to be ineffective in isolating the complexes in pure form from membranes of *T. pyriformis* mitochondria and *P. denitrificans*. Extraction of the energy conserving membranes covalently bound with radioactive N_3CCP with $CHCl_3/CH_3OH$ (2 : 1, v/v), a procedure which gives essentially quantitative removal of the labeled proteolipid in animal mitochondria, gave only about 30% removal of radioactivity into the organic phase. SDS-polyacrylamide gel electrophoresis of the residual pellets after $CHCl_3$-CH_3OH extraction did not show any single radioactive peptide. Extraction with $CHCl_3/CH_3OH$ evidently disrupts the membrane in such a way that the radioactively labeled protein is partially degradated to products of non-uniform sizes, possibly by proteases present in the membrane which are active during relatively long (16 h) extraction procedure [8].

Discussion

Earlier studies of uncoupler interaction with the mitochondrial membrane [1,2,9] give results consistent with the present observations using the energy conserving membranes from *P. denitrificans* and *T. pyriformis*. A high affinity uncoupler binding protein site of similar apparent molecular weight is present in all three energy conserving membranes, and in each case binding to this site directly correlates with uncoupling activity. Azidonitrophenol has also been reported to bind to a specific site in the mitochondrial membrane and photolysis is reported to result in incorporation into peptides with apparent molecular weights of 55 000, 30 000 and 9000 [10—12]. Hanstein and Hatefi [10,11] have concluded that the primary site is the 30 kdalton peptide while Kurup and Sanadi [12] suggest that the primary site is the 9 kdalton peptide. It appears very probable that the 10—15 kdalton proteolipid labeled by N_3CCP (Ref. 2 and this paper) is the same as the 9 kdalton peptide labeled by the azidonitrophenol and that this is the primary uncoupler binding site.

In summary: Uncouplers bind to a specific proteolipid of apparent molecular weight of 10 000—15 000 not only for animal mitochondria but also for mitochondria of *T. pyriformis* and membranes from *P. denitrificans*. In each case

uncoupling activity directly correlates with uncoupler binding to this site. The presence of a unique uncoupler binding site in these widely different organisms indicates the presence of a common mechanism of uncoupling and a key role of the uncoupler binding proteolipid in ion transport and, or oxidative phosphorylation.

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