A SPIN-LABEL CARBODIIMIDE AS A PROBE FOR MITOCHONDRIAL ATPase

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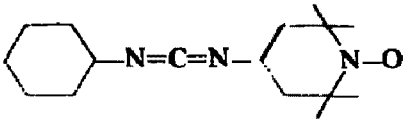
1. Introduction

Studies on the mechanism of ATP synthesis in mitochondria have mainly utilized two different approaches. The first consists in the separation of membrane components and their reassembly [1]. The second is based on the utilization of intrinsic or extrinsic spectroscopic probes [2, 3] for the study of environment, distances and interactions of different membrane components. Along this line, derivatives of anilinonaphthalene sulfonic acid have been successfully employed for the detection of generalized structural changes in the mitochondrial membrane during energy conservation [4]. Similarly, fatty acids, spin-labelled at different distances from the carboxyl group have reported on several physiochemical parameters [5] of biological membranes.

The inhibitors antimycin [6] and aurovertin [7, 8] have been utilized as probes for specific regions of the mitochondrial membrane, namely the coupling site II region and that of the ATPase. Unfortunately, the interpretation of the fluorescence data concerning the interaction of these inhibitors with the mitochondrial membrane is difficult, due to the scare knowledge of the physicochemical parameters affecting the fluorescence of these compounds. In the present study the synthesis of a spin-label carbodiimide (NCCD*) is described.



It appears a good inhibitor of mitochondrial ATPase, and due to its interpretable spectral characteristics [3], can be utilized as a probe for studying the environment and the interaction of this enzyme. In particular, interaction of NCCD with a site of low viscosity would give rise to a resonance spectrum with three sharp lines of nearly equal height characteristic of freely tumbling nitroxide molecules [5]. Unequal broadening of these lines would be instead characteristic of immobilized nitroxide molecules [5], and indicate that there are limitations in the rotational mobility of the probe at this binding site. Moreover, the hyperfine coupling constant of the nitroxide is 1 or 2 G smaller in hydrocarbon solvents than in water and may permit polarity measurements at the inhibitory site. Finally, since the magnetic environment of a nitroxide is perturbed by the presence of nearby free radicals or paramagnetic species, distances between the NCCD site and an ATP–Mn^{2+} binding site may be calculated.

2. Materials and methods

4-Amino-2,2,6,6-tetramethyl-piperidin-1-oxyl (I) was obtained by a published method [9].

Cyclohexylisothiocyanate (II) was purchased from Schuchardt Chemiske Fabrik, Germany.

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Abbreviations:
NCCD, *N*-(2,2,6,6-tetramethyl-1-oxypiperidyl)-*N'*-(cyclohexyl)-carbodiimide; DCCD, *N, N'*-dicyclohexylcarbodiimide.

The synthesis of NCCD was carried out following in part the method of K[illegible]arev and Knorre for the preparation of a soluble spin-label carbodiimide [10].

N-(2,2,6,6-tetramethyl-piperidyl-1-oxyl)-N'-(cyclohexyl) thiourea (III) was prepared by adding to a solution of 0.01 moles of I in 15 ml of absolute ether, a three fold molar excess of II dissolved in 15 ml of absolute ether. After 12 hr at room temp. 1.3 g of yellow crystals (41%) separated off. After washing with petroleum ether the product was used without further purification.

N-(2,2,6,6-tetramethyl-1-oxylpiperidyl)-N'-(cyclohexyl) carbodiimide (IV, NCCD) was prepared by dissolving 1.3 g of III in 50 ml of anhydrous benzene and 25 ml of anhydrous pyridine.

3 g of freshly precipitated yellow mercuric oxide were added and the mixture was boiled with stirring for 60 min. The solution was filtered hot. The solvent was evaporated under reduced pressure and the residual red oil was extracted with petroleum ether until no coloration appeared in the ether phase. The solution was concentrated and left at room temp. until crystallization occurred. The red crystals were filtered off and the filtrate was concentrated to obtain more product. The two fractions, pooled together, and recrystallized from petroleum ether yielded 650 mg of product: Yield 50%, m.p. 64–65°. $C_{16}H_{28}N_3O$ requires C 69.0%, H 10.08%, N 15.1%. Found C 68.0%, H 10.1%, N 14.6%. The IR spectrum had a maximum at 2120 cm^{-1} corresponding to the –N=C=N– stretching [11].

Mitochondria from beef heart were prepared according to a published method [12]. Mitochondrial fragments were prepared according to Beyer [13]. ATPase was measured according to Chance and Nishimura [14], with a Beckman Expandomatic pH meter connected with a potentiometric recorder (Rikadenki). Electron paramagnetic resonance spectra were obtained in Varian E3 spectrometer, at 25°.

3. Results and discussion

The inhibition by NCCD of ATP hydrolysis catalyzed by mitochondrial fragments as a function of time is presented in fig. 1. NCCD (1 nmole/mg protein) was added at 0° to a suspension of mitochondrial fragments (20 mg/ml). As a control, an identical suspension of membranes was kept for the same time at 0° in the absence of inhibitor. After different incubation periods, aliquots of the suspension were withdrawn and assayed in a buffer containing 100 mM KCl, 2 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 μM FCCP. The final protein conc. was 0.2 mg/ml. Addition of 0.8 mM ATP produced the characteristic pH decrease. The ratio (×100) between the rate of pH

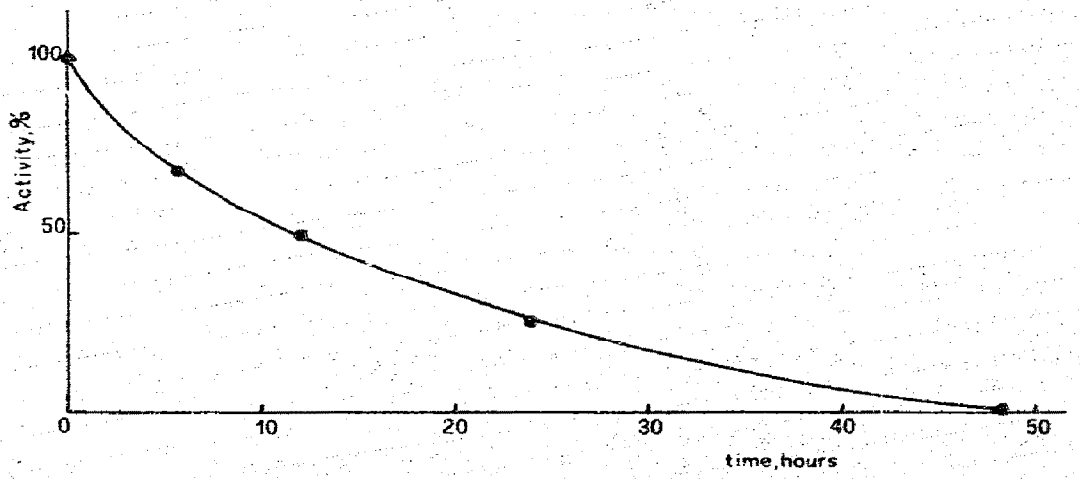

Fig. 1. Time dependence of the inhibition of mitochondrial ATPase by NCCD. Beef-heart mitochondrial fragments (30 mg protein per ml) were incubated at 0° in the presence and absence of 1 nmole NCCD per mg protein in 0.25 M sucrose and 5 mM Tris. After a given time samples of 0.6 mg were withdrawn and the rate of ATP hydrolysis measured (see Methods). The ratio inhibited/uninhibited velocity (×100) is plotted versus the time of preincubation. The incubation mixture contained: 0.1 M KCl, 2 mM Tris-HCl, pH 7.5, 1 μM FCCP and 5 mM $MgCl_2$. Vol 3 ml. The uninhibited rate of ATP hydrolysis was 0.15 μmoles/min/mg protein.

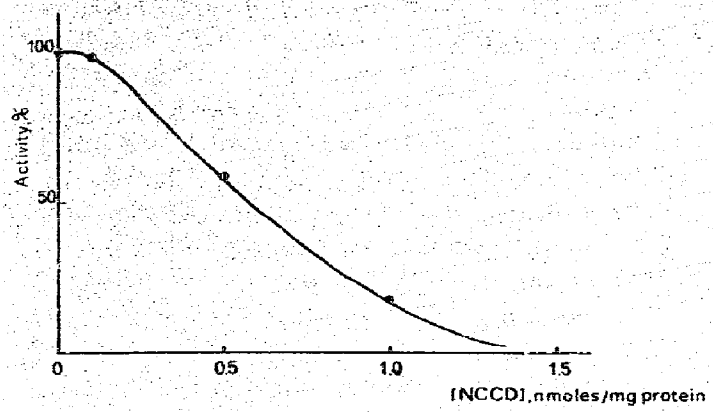

Fig. 2. Concentration dependence of the inhibition of mitochondrial ATPase by NCCD. Experimental conditions were as in fig. 1. The incubation of membrane fragments with the inhibitor (at different concentrations) lasted 48 hr.

change in the inhibited and uninhibited samples is plotted in the diagram of fig. 1, as a function of the incubation period. Complete inhibition was reached after 48 hr. After this time the ATPase activity of the controls was not significantly decreased. The time dependence of NCCD inhibition resembles that of DCCD [15]. In fig. 2 the inhibitory effect of different concentrations of NCCD on the mitochondrial ATPase was measured under the same experimental conditions described above, after 48 hr of incubation with the inhibitor at 0°. It appears that 85% inhibition occurs at a concentration of 1 nmole NCCD/mg protein, which is the same order of magnitude as the concentration of cytochromes in the membrane or the number of sites titrated by inhibitors such as antimycin, oligomycin and DCCD [16].

The above data suggest that, despite the higher polarity of NCCD in comparison with DCCD, the inhibition of ATPase still occurs at low concentrations. However, the time course of the inhibition appears to be several fold faster with DCCD than with NCCD, under the same experimental conditions.

In fig. 3 is presented the electron paramagnetic resonance spectrum of mitochondrial membrane fragments incubated in the presence of 1 nmole NCCD/mg protein as described above for 48 hr. 1 mM K-ferricyanide was also present in the preincubation medium in order to prevent the reduction of the spin label and

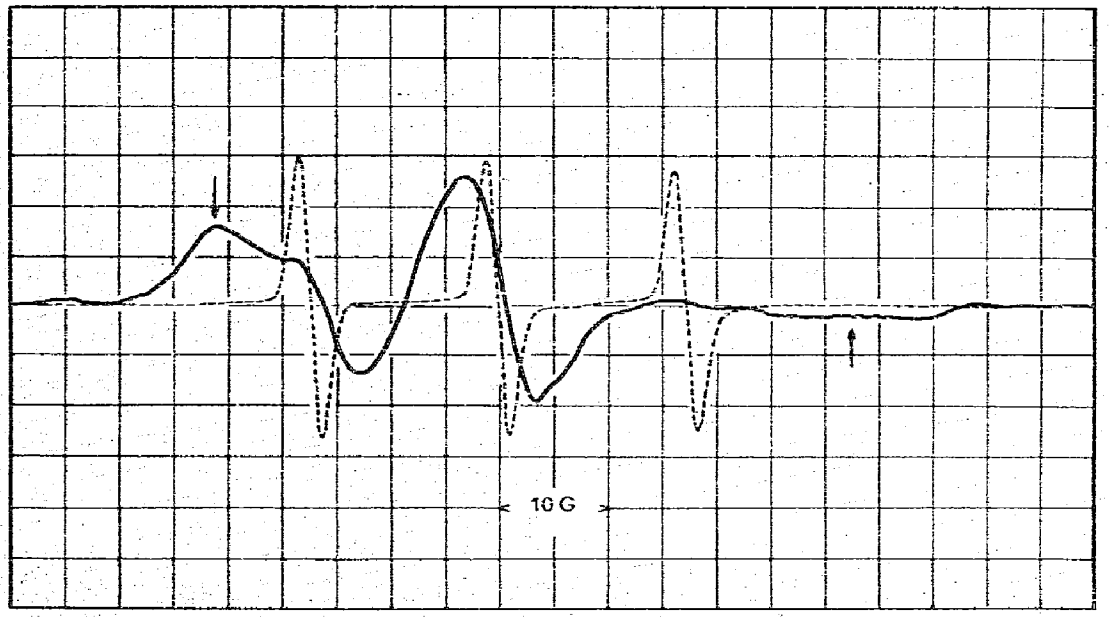

Fig. 3. Electron paramagnetic resonance spectrum of NCCD bound to mitochondrial fragments. Experimental condition as in fig. 1. After preincubation with NCCD (1 nmole/mg protein) the fragments were dialyzed against sucrose-Tris buffer until the amount of paramagnetic resonance signal associated with the spin-labelled membranes remained constant. The spectrum (solid line) was taken in a Varian 3 spectrometer, at 25° in a 2 mm cuvette. Protein concentration was 30 mg/ml. The spectrum reproduced with a dotted line was obtained from a solution of NCCD in water. The concentration of the free (30 μM) and bound NCCD were the same, but the spectrum of the latter was recorded at a sensitivity 10 times higher than that of the former.

the consequent disappearance of the paramagnetic resonance signal.

The samples were further dialyzed for several hours to eliminate any free inhibitor.

In fig. 3, the paramagnetic resonance spectrum of NCCD in sucrose-Tris buffer is also shown (dotted line). The concentration of the inhibitor was the same as for the spectrum in the presence of membranes (solid line), but the spectrum of the free species was taken at an instrumental sensitivity 10 times lower than the spectrum of the bound species. The double integrals of the two spectra (i.e. the number of spins present in the samples) agreed within 15% experimental error. This indicates that little if any inhibitor was destroyed under our experimental conditions.

The three sharp lines of the spectrum of NCCD in sucrose-Tris buffer are the consequence of the rapid isotropic motion of the molecule, with averaging of all anisotropic interactions.

The correlation time of free NCCD computed according to conventional techniques is 1.7×10^{-11} sec. The spectrum of bound NCCD has instead lines unequally broadened. The two outer peaks, marked by arrows, appearing in this spectrum, are the consequence of the slow tumbling motion of the label. They are separated by 58 G. The larger the separation of the two peaks, the lesser is the motional freedom of the spin label. Nitroxide in water has in fact a separation of the outer lines of 36 G while in a solid powder spectrum this separation becomes 64 G. From the comparison of the spectrum of membrane bound NCCD with those obtained by computer simulation [5] the correlation time of the bound inhibitor appears to be close to 1×10^{-8} sec. The spectral analyses reported above indicate that the site of binding of NCCD imposes remarkable limitations on the molecular motion of the inhibitor.

The hyperfine splitting constant of NCCD in water was 16.8 G, while in the case of the inhibitor bound to the membrane it was approx. 1 G smaller. In the latter case the constant was calculated from the equation $a = (1/6)T_0 + (1/3)T_i$, were T_0 and T_i are the distances in G between outer and inner peaks, respectively. The polarity of the site of NCCD binding on the mitochondrial membrane appears therefore to be slower than that of water.

It appears in conclusion that NCCD binds to the inhibitory site of mitochondrial ATPase, approximately in stoichiometric concentration. This site has characteristics of low polarity and is such to impose a strong constraint upon the rotational motion of the inhibitor.

The spectrum of NCCD in lecithin dispersion [17], obtained under the same conditions described for membrane fragments, was that of a very slightly immobilized radical species (correlation time of 1×10^{-10} sec) as one would expect if the probe penetrated deeply into the highly disordered region of hydrocarbon chains in the depth of the membrane [3]. The comparison of the spectra of NCCD in membrane fragments and phospholipid vesicles may indicate that the binding site of NCCD in mitochondria has not the characteristics of a lipid.

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