Site-directed Spin Labeling of the Mitochondrial Membrane

SYNTHESIS AND UTILIZATION OF THE ADENOSINE TRIPHOSPHATASE INHIBITOR (N-2,2,6,6-
TETRAMETHYL-PIPERIDYL-1-OXYL)-N’-(CYCLOHEXYL)-CARBODIIMIDE

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SUMMARY

NCCD (N-(2,2,6,6-tetramethyl-piperidyl-1-oxyl)-N’-(cyclohexyl)carbodiimide) is a spin label inhibitor of ATPase of mitochondrial membrane fragments. Upon binding (~0.5 nmole per mg of protein) its electron paramagnetic resonance spectrum becomes highly immobilized (τo = 2.10 × 10⁻⁸ s). The bound but not the free label is reduced by succinate, indicating that electrons can be transferred from the respiratory chain to the ATPase system. The Mn⁺⁺ATP complex decreases the paramagnetic signal of NCCD bound to membrane fragments about 30%. Such an interaction can be the consequence of the vicinity of the binding sites of the two species.

ATP synthesis and hydrolysis in mitochondria is catalyzed by enzymes intimately associated with the membrane structure (1–3). The number of membrane components and their complex interactions have limited the use of direct spectroscopic methods for the detection of structural informations on biological membranes. Alternatively the use of fluorescent probes has afforded a very sensitive and flexible technique which has shown changes in the mitochondrial membrane structure associated with energy conservation (4–6). Similarly, spin labels (7, 8) have yielded valuable information concerning the thermal agitation of polymethylene chains of membrane phospholipids and their temperature-induced transitions (9, 10). Specific spin labeling with androstanolone (11) and of cytochrome c with iodoacetamide nitroxide (12, 13) are examples of site-directed labels (14, 15) able to explore a specific region of the membrane. The use, as a probe, of antimycin, an inhibitor of the electron transport chain of mitochondria, has proved very useful in elucidating the characteristics of the portion of the mitochondrial respiratory chain in connection with the second site of energy conservation (16). Another mitochondrial inhibitor, aurovertin, has been utilized as a probe for F₁. In fact, it reacts with membrane-bound mitochondrial ATPase forming a highly fluorescent derivative (17, 18).

This study will describe the synthesis and some characteristics of a spin label inhibitor of mitochondrial ATPase, namely N-(2,2,6,6-tetramethyl-piperidyl-1-oxyl)-N’-(cyclohexyl)-carbodiimide.

It is an analog of another inhibitor of mitochondrial ATPase, dicyclohexylcarbodiimide (19, 20) and conserves almost intact its inhibitory properties. Moreover its electron paramagnetic resonance spectrum is easily interpretable. Theories (21–24) are, in fact, available which quantitatively correlate molecular motion, environmental polarity and the distance from nearby paramagnetic centers with the electron paramagnetic resonance spectra of nitroxide. NCCD, dissolved in a polar environment of low viscosity, has a characteristic spectrum with three sharp, almost identical resonances separated by 16.8 G. Slow isotropic motion of NCCD, such as in glycerol at −40°C, results in unequal broadening of the resonances already present in water and the appearance of two new resonances at the outer extremes of the spectrum. The separation of the resonances in a solvent of low polarity, such as chloroform, becomes smaller due to a decrease of the hyperfine splitting constant from the value of 16.8 G in water to that of 15.4 G. Similar to other nitroxides, the electron paramagnetic resonance spectrum of NCCD is affected by the presence of other paramagnetic species at a close distance (25, 26). Dipolar interaction of Mn⁺⁺ ions with NCCD in a rigid lattice results in a decrease in the amplitude of the spectrum without broadening. From such a decrease, the distance between the two interacting species can be calculated (24).

This study will describe the synthesis of NCCD, its inhibitory properties in membrane fragments, and its binding to mitochondrial ATPase and phospholipid dispersions. Moreover the interaction of oxidation-reduction components of the respiratory chain with the label will be described as well as the influence on the spectrum of NCCD of the presence of the Mn⁺⁺ATP complex in the membrane.

METHODS AND MATERIALS

Synthesis of Spin Label

4-Amino-2,2,6,6-tetramethyl-piperidin-1-oxyl (Compound I) was obtained by a published method (27), and cyclohexylisothio-

1 The abbreviations used are: NCCD, N-(2,2,6,6-tetramethyl-piperidyl-1-oxyl)-N’-(cyclohexyl)carbodiimide; DCCD, N,N’-dicyclohexyl carbodiimide.
cyanate (Compound II) was purchased from Schuchard Chemische Fabrik, Germany. The synthesis of the spin label carbodiimide was partly derived from the method of Kumarev and Knorre (28).

\[ \text{N-}[2,2,5,5\text{-tetramethyl-1-oxylpiperidyl}]-\text{N'}-[\text{cyclohexyl} \text{thio}- \text{urea (Compound III)}] \]  

A solution of 0.01 mole of Compound I in 15 ml of absolute ether was mixed at room temperature with a 3-fold molar excess of Compound II in 15 ml of absolute ether. After 12 hours at room temperature 1.3 g of yellow crystals separated off were washed with petroleum ether; yield was 41%.

\[ \text{N (2,2,5,5-Tetramethyl-1-oxylpiperidyl) - N' (cyclohexyl) carbodiimide (Compound IV)} \]  

Compound III (1.3 g) dissolved in 50 ml of anhydrous benzene and 25 ml of anhydrous pyridine was heated with 3 g of freshly precipitated yellow mercuric oxide, and the mixture was boiled with stirring for 60 min. The solution was filtered hot and the filtrate was concentrated and left at room temperature until crystallization occurred. The red crystals were filtered off and the filtrate was concentrated to obtain more product. After recrystallization of both fractions together from petroleum ether Compound IV was obtained with m.p. 64-65°C, yield 50%. Chemical analysis yielded the following percentage composition:

- Calculated: C 69.0, H 10.1, N 15.1
- Found: C 68.4, H 10.1, N 14.9

Infrared spectra (in chloroform) had a band at 2120 cm\(^{-1}\) corresponding to the N==C==N stretching (29). The stability of the diamide was found excellent if the substance was kept at low temperature and dry. An attempt was initially made to synthesize \( \text{N-}[2,2,5,5\text{-tetramethyl-1-oxylpiperidyl}]-\text{N'}-[\text{cyclohexyl} \text{carbodiimide}, \text{using essentially the procedure described above. However the compound underwent quick decomposition according to the theory developed by Kivelson (21) by the following equation derived by Stone et al. (22).} \]

\[
\frac{1}{T_i(0)} - \frac{1}{T_i(0)\pm 1} = \left( \frac{B}{8} \right) + \left( \frac{4}{15} \right) \beta g B \tau_i. \tag{1}
\]

The experimentally measured parameters of this equation are:

\[
\frac{1}{T_i(0)} = 2.8 \times 10^{-10} \times \beta g B \tau_i.
\]

\( b = \beta A/3(\tau_i - 1) \)

where \( A \) is the hyperfine coupling constant along the z axis and \( B \) is that along the x or y axis (symmetry is assumed around the axis of the applied magnetic field). Their values are \( A = 87 \) MHz and \( B = 14 \) MHz.

\[
\delta H = \text{peak to peak separation in G of the central resonance}
\]

\[
\delta H = \frac{1}{T_i(0)} - \frac{1}{T_i(0)\pm 1} = \left( \frac{B}{8} \right) + \left( \frac{4}{15} \right) \beta g B \tau_i. \tag{1}
\]

The distance between two unlike spin labels was calculated according to the theory developed by Leigh (24). In a rigid lattice the line width of the paramagnetic resonance lines of a nitroxide radical perturbed by the magnetic field of a nearby paramagnetic species such as Mn\(^{2+}\) is given by

\[
\delta H = C(1 - 3 \cos^2 \theta_E) + \delta H_0 \tag{2}
\]

where \( \delta H_0 \) is the residual line width of the label in the absence of Mn\(^{2+}\), \( \theta_E \) is the angle between the applied magnetic field and the vector joining the two spins and

\[
C = \frac{g^2/2 \mu^2}{r^5 H^2}. \tag{3}
\]

where \( r \) is the distance between the two unlike spins, \( \mu \) is the magnetic moment of the paramagnetic species, \( g \) is \( \sqrt{2} (g_2 + g_2) \) and \( \tau_i \) is the correlation time for the dipolar interaction, i.e. the electron spin relaxation time of Mn\(^{2+}\). \( C \) has been calculated by appropriate computer simulation of the interaction between the two unlike spins at different distances and angles (24, 26).

**RESULTS**

**Inhibition by NCCD of ATP Hydrolysis in Mitochondrial Fragments**—Significant inhibition of the initial rate of ATP hydrolysis by NCCD could be obtained either at high concentrations of the inhibitor or by preincubating low concentrations of the inhibitor for several hours at 0-4°C with mitochondrial fragments. Similarly to NCCD, DCCD has been previously described to exert its inhibition in a time-dependent reaction (20).

In the experiment reported in Fig. 1A, mitochondrial fragments were measured as a pH decrease according to the method of Chance and Nishimura (34) or from the P1 liberated according to Fiske and SubbaRow (35).

**Calculations**

The correlation time of spin labels (\( \tau_i \)) was calculated according to the theory developed by Kivelson (21) by the following equation derived by Stone et al. (22).

\[
\frac{1}{T_i(0)} - \frac{1}{T_i(0)\pm 1} = \left( \frac{B}{8} \right) + \left( \frac{4}{15} \right) \beta g B \tau_i. \tag{1}
\]
membrane fragments as described. In Fig. 1A the rate of pH change in the absence of inhibitor and that at different times of incubation with NCCD are plotted in Fig. 1 in the presence of 1 nmole of NCCD per mg of protein. The rate of the uninhibited samples was 0.15 µmole per min per mg of protein, and was not significantly decreased upon aging.

In Fig. 2A is reproduced the paramagnetic resonance spectrum of NCCD in water (Fig. 2A) has three narrow lines almost identical in amplitude. These resonances are separated by 16.8 G. From the relative intensities of the central and high field lines, the value of the magnetic field of the central resonance and its width in G, the correlation time of the spin label can be calculated according to Equation 1 and has a value of 1.7 × 10⁻¹¹ s.

In Fig. 2B is reproduced the paramagnetic resonance spectrum of NCCD in the presence of mitochondrial membranes. The sample utilized for this experiment was treated as described above with 1 nmole of NCCD per mg of protein (mitochondrial fragments) for 48 hours in the presence of 1 mM ferricyanide, and finally dialyzed. Ferricyanide prevented the reduction of the label, often seen in membranes, with loss of EPR signal. Table I reports the values of the double integrals of the spectra of free and bound NCCD. The EPR spectrum of the undialyzed material was a combination of free and bound NCCD, in which the free form accounted for 48% of the total signal (cf. Table I).

The spectrum of bound NCCD has, only two small shoulders in place of the low and high field resonances of the free inhibitor. The central line is broadened and two new resonances (marked by arrows) appear at the outer extremes of the spectrum, separated by 58 G. Since Equation 1 cannot be utilized under the conditions of strong immobilization of the spin label, an approximate value for τc was calculated from the comparison with published spectra of similar shape obtained by computer simulation using different values of τc (18). In this way the value of τc for NCCD bound to mitochondrial fragments was estimated to be approximately 2 × 10⁻¹¹ s. The spectrum of membrane-bound NCCD appears to be very similar to that obtained in glycerol at -20°C (Fig. 3).

Effect of Inhibitors and Substrates on Electron Paramagnetic Resonance Spectrum of NCCD Bound to Mitochondrial Fragments

The experiments reported in Table I were performed in order to establish whether NCCD radicals were reduced (with loss of EPR signal) during the incubation of mitochondrial fragments.
I that reduced ubiquinone was able to induce a disappearance of the EPR spectrum of NCCD dissolved in ethanol.

When mitochondrial fragments were incubated with DCCD (1 nmole per mg) or oligomycin (2 µg per mg) prior to the addition of NCCD, 16% and 90% of the integrated EPR signal due to bound NCCD was recovered, respectively. While the experimental error and that introduced by the double integration do not allow to consider significant the change promoted by oligomycin, it can be concluded that DCCD largely prevents binding of NCCD to the mitochondrial membrane fragments.

**Electron Paramagnetic Resonance Spectra of NCCD in Phospholipids**—Two of the three major phospholipid components of the mitochondrial membrane (38), namely phosphatidylethanolamine (40%) and diphosphatidylglycerol (20.4% of total mitochondrial lipids), were tested as possible sites of NCCD binding. Such an experiment was also suggested by the finding that a proteolipid appears to be the binding site of DCCD (39). Microdispersions of the phospholipid (see “Methods and Materials”) were prepared and incubated with NCCD at 0° for 48 hours. The samples were not dialyzed.

In Fig. 4A the EPR spectrum of NCCD bound to phosphatidylethanolamine (30 µm and 2.6 mg per ml, respectively) is shown. The three resonances of this spectrum are only slightly unequal and broadened, with respect to the spectrum of Fig. 2A. The correlation time calculated from Equation 1 is $1.2 \times 10^{-9}$ s. The spectrum was taken under the conditions described for phosphatidylethanolamine and is very similar to that of NCCD in diphosphatidylglycerol.

**Effect of Mn**++**ATP and Mn**++ADP**++ Complexes on Electron Paramagnetic Resonance Spectrum of NCCD in Mitochondrial Fragments**—The substrate for mitochondrial ATPase has been shown to be the 1:1 complex between ATP and a divalent metal cation (40). We have confirmed this by using Mn**++**ATP complex as the substrate of the ATPase of beef heart mitochondrial fragments. In fact maximum hydrolysis occurred when the stoichiometry between ATP and Mn**++** was 1:1 and decreased when free ATP was present in excess.

Addition of Mn**++ATP complex (5 mm) to NCCD-labeled mitochondrial fragments results in a decrease of the amplitude of the EPR resonances of NCCD spectrum without apparent broadening or distortion (Fig. 5). No decrease of either amplitude or width of the resonances was observed when Mg**++ATP complex (5 mm), free MnCl₂ (5 mm) or MgCl₂ (5 mm) were used. The spectrum of NCCD in the presence of Mn**++** complexes was corrected by subtracting, point by point, the spectrum of the paramagnetic species alone in the membrane which gives a broad background.

In Fig. 5, the spectrum of NCCD-labeled membranes and that obtained in the presence of Mn**++ATP are shown. The decrease in amplitude of the central line is 35%, which can be interpreted as a dipolar interaction between the two paramagnetic species in a rigid environment. The addition of Mn**++ADP complex (5 mm) to NCCD-labeled membranes gave a very similar (28%) decrease in amplitudes amplitude without broadening.

**DISCUSSION**

It has been shown that most water-insoluble carbodiimides bind irreversibly and stoichiometrically to some membrane component (possibly a proteolipid) which results in inhibition of ATP syn-
FIG. 4. Electron paramagnetic resonance spectra of NCCD bound to lecithin and cardiolipin microdispersions. The dispersions of phospholipids, obtained by ultrasonic treatment of samples dried under nitrogen stream and suspended in 0.25 M sucrose and 5 mM Tris-HCl, pH 7.5, (see "Methods and Materials" for further details), contained 2.6 mg per ml of lecithin and 3.2 mg per ml of cardiolipin, respectively. Incubation of the lipid with NCCD (30 \( \mu \)M) as well as the spectral analysis was carried out as described for mitochondrial fragments in Figs. 1 and 2. Dialysis was omitted in the case of phospholipid, since during this procedure all EPR signal was lost.

FIG. 5. Electron paramagnetic resonance spectra of NCCD bound to mitochondrial fragments in the presence and absence of Mn\(^{++}\). Binding of NCCD and EPR spectra were performed according to Figs. 1 and 2, using 1 n mole of NCCD per mg of protein. Mn\(^{++}\)-ATP complex was added at a concentration of 6.0 mM. The spectrum of Mn\(^{++}\)-ATP complex in the absence of NCCD was subtracted point by point from that in the presence of the inhibitor. Solid line NCCD bound to membranes. The inset shows the EPR spectra of Mn\(^{++}\)-ATP complex in membrane fragment appearing as a base-line drift in this magnetic field interval, and that of Mn\(^{++}\)-ATP complex plus NCCD. The difference between the two spectra is plotted as a dotted line in the figure.

The correlation time of NCCD in mitochondrial membrane fragments is of the order of \( 10^{-8} \) s, indicating binding to a fairly "rigid" site, which might be compared to a solution of glycerol at \(-20^\circ\), having a viscosity of \( 1.34 \times 10^{-2} \) poise. Such a value appears to be different from that obtained with another inhibitor of ATPase, namely aurovertin, whose fluorescence spectrum in the membrane of mitochondrial fragments can be compared with that in glycerol at \( 17^\circ \). It is thus probable that NCCD and aurovertin sites are different. A decrease of the NCCD hyperfine coupling constant in membrane fragments with respect to that in water indicates that the site of NCCD binding has characteristics of low polarity.

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A. Azzi and P. Graziotti, unpublished data.
Both parameters, polarity and mobility of the membrane-bound probe, are not affected by the presence of ATP or ADP, either alone or as Mg++ complexes, indicating that no conformational changes due to nucleotide binding are measurable at the inhibitor binding site. However Mn++-adenine nucleotide complexes modify the EPR spectrum of membrane-bound NCCD. Decrease in the resonances amplitude of a nitroxide spectrum without line broadening due to dipolar interaction with Mn++-ATP complex has been described (24). The theory proposed which permits distance calculations between nitroxide and Mn++ requires that the two interacting systems are embedded in a rigid lattice, a condition which appears to be satisfied in our case, where the nitroxide label has a correlation time around $10^{-8} \text{s}$. Unfortunately the correlation time of the dipolar interaction, which in this case is the electron spin relaxation time of Mn++-ADP or -ATP complex, is not available in mitochondria. However, reasonable guesses can be made since in most proteins it varies between $10^{-9} \text{s}$ and $10^{-7} \text{s}$. If these values are assumed for the correlation time of the interaction, the distance between Mn++ or Mg++ and ATPase complex appears to be of the order of 20 A. It is in fact known that Mg++ or Mn++ can activate the hydrolysis of ATP and that such an effect is not promoted if an excess of free ATP is present, indicating that the complex of Mg++ or Mn++ nucleotide can serve as the substrate for ATPase (40).

Since the interaction of Mn++ with the label occurs without distortion, the two species must lay within an angle of approximately 20° (24, 25). Interaction of bound NCCD with some ATP is present, indicating that the complex of Mg++ or XIn++-ATP and that such an effect is not promoted if an excess of free ATP is present, indicating that the complex of Mg++ or Mn++-adenine nucleotide can serve as the substrate for ATPase (40).

The chemical nature of the site of binding of DCCD (and NCCD) is of interest, since the simplest membrane components which binds DCCD is a proteolipid (39). The data reported above suggest that NCCD binds to some protein component of the membrane. In fact NCCD bound to mitochondria has an EPR spectrum with characteristics which are very different from those in phospholipid microdispersions. There is however the possibility that phospholipid-protein interaction may produce strong immobilization of fatty acid hydrocarbon chains (41) and of the associated NCCD. However since detachment of the probe cannot be obtained in the membrane by extensive dialysis but is very easy in phospholipid dispersions, some protein component of the membrane appears to be the most probable candidate as the site for NCCD binding.

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