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Fluorescent Analogues of *N,N'*-Dicyclohexylcarbodiimide as Structural Probes of the Bovine Mitochondrial Proton Channel†

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ABSTRACT: *N*-Cyclohexyl-*N'*-[4-(dimethylamino)-α-naphthyl]carbodiimide (NCD-4) and *N*-cyclohexyl-*N'*-(1-pyrenyl)carbodiimide (NCP) are two novel fluorescent analogues of the mitochondrial inhibitor dicyclohexylcarbodiimide (DCCD). Although nonfluorescent in aqueous media, both compounds form fluorescent conjugates with mitochondrial electron transport particles (ETP_H) or purified H^+-ATPase (F_1-F_0) vesicles. DCCD prevents the reaction of ETP_H with both NCD-4 and NCP. The fluorescent probes are effective inhibitors of ATPase activity and ATP-driven membrane potential, although their reaction rates are considerably slower than that of DCCD. The fluorescence of NCD-4- or NCP-treated H^+-ATPase is quenched by hydrophobic spin-label nitroxide derivatives of stearic acid (*x*-NS) in the order 16-NS > 12-NS > 7-NS ≃ 5-NS, whereas membrane-impermeant iodide ions have negligible effect. The quenching behavior of 16-NS (the most effective quencher) suggests that a small fraction of labels remain inaccessible to the quencher. It is concluded that the DCCD-binding sites are oriented toward the membrane lipids and are located in the lipid bilayer ca. 18 Å from the membrane surface.

The mitochondrial inhibitor dicyclohexylcarbodiimide (DCCD)[1] is a highly specific inhibitor of ATPase activity and ATP-driven proton translocation in both eukaryotic and prokaryotic H^+-ATPases (Catell et al., 1971; Linnet & Beechey, 1979). In all cases, the mechanism of inhibition involves the selective modification of a glutamic acid residue (aspartic in *Escherichia coli*) in the so-called proteolipid subunit of the H^+-ATPase complex (Sebald et al., 1980; Sebald & Wachter, 1980; Hoppe et al., 1980).

The proteolipid, or DCCD-binding protein, is a hydrophobic protein of molecular weight ca. 8000. It forms part of the membrane sector (F_0) of the H^+-ATPase (F_1-F_0) and is believed to function as the proton-conducting element or channel (Criddle et al., 1977; Moran et al., 1980; Celis, 1980). In mitochondrial and chloroplast F_0, there are probably six copies of the proteolipid (Sigrist-Nelson et al., 1978; Sebald et al., 1979) although the modification of only one subunit is sufficient for complete loss of ATPase activity (Sebald et al., 1976; Graf & Sebald, 1978), suggesting that the six monomers function in a concerted manner (Friedl et al., 1980).

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[1] Abbreviations: DCCD, dicyclohexylcarbodiimide; ATPase, adenosinetriphosphatase; NCD-4, *N*-cyclohexyl-*N'*-[4-(dimethylamino)-α-naphthyl]carbodiimide; NCP, *N*-cyclohexyl-*N'*-(1-pyrenyl)carbodiimide; ETP_H, mitochondrial electron transport particles; oxonol VI, bis(3-propyl-5-oxoisoxazol-4-yl)-2,4-pentadienylideneoxonol; *x*-NS, *x*-(4,4-dimethyl-*N*-oxyoxazolidin-3-yl)stearic acid (*x* = 5, 7, 12, 16); TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; NADH, reduced nicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane.

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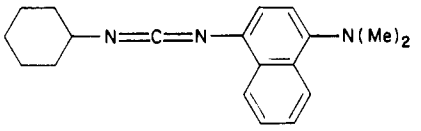
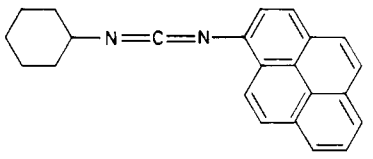
To understand the molecular basis of proton translocation, it is important to know the manner in which the proteolipid monomers are arranged within the mitochondrial membrane and the location of the functional group that binds DCCD to render the proton channel inactive. Such studies have been hampered by the lack of suitable site-specific spectroscopic probes, although a spin-label analogue of DCCD has provided evidence that in chloroplast membranes, individual proteolipid monomers are separated from each other by 15–20 Å (Sigrist-Nelson & Azzi, 1979). The fluorescent probe NCD-4



NCD-4



NCP

recently introduced by Chadwick & Thomas (1983) is also an analogue of DCCD. It has been used to probe the Ca^{2+} binding site in sarcoplasmic reticulum Ca^{2+}–ATPase (Chadwick & Thomas, 1983, 1984). We report here that this reagent and a novel pyrene derivative of DCCD, which we shall designate NCP, are both similar to DCCD in their inhibitory properties of the mitochondrial H^+–ATPase and, further, that fluorescence quenching experiments can provide useful information on the location of the DCCD-binding site of the proteolipid.

Materials and Methods

Bovine ETP_H were isolated from heart mitochondria by the method of Linnane & Ziegler (1958). Lysolecithin extraction of the particles afforded purified H^+–ATPase vesicles (F_1–F_0) according to the procedure of Hughes et al. (1982). The ATPase activity was measured by incubating the enzyme with ATP for 10 min at 30 °C (Tzagoloff et al., 1968) and assaying the inorganic phosphate produced (Fiske & Subbarow, 1925). Membrane potential changes due to ATP or NADH energization were measured indirectly with the voltage-sensitive dye oxonol VI (Pringle & Sanadi, 1984) by monitoring the time-resolved changes in optical absorbance, $\Delta A_{594-630}$, on a dual wavelength spectrophotometer (Perkin-Elmer 557). Assays were carried out in 2 mL of Tris–acetate (40 mM, pH 7.5)/sucrose (250 mM). Fluorescence quenching experiments were carried out on a Perkin-Elmer MPF-44A spectrofluorometer. All spectra were recorded at 20 °C and are uncorrected. Unless otherwise specified, preparations of ETP_H or purified H^+–ATPase were diluted to 2 mg of protein/mL in Tris-HCl (75 mM, pH 7.5)/sucrose (250 mM) for incubation with inhibitors. Further experimental details will be given in the appropriate figure legends.

Oxonol VI was a gift from Drs. B. Chance and L. Bashford. Spin-labeled stearic acids (*x*-NS) were kindly donated by Dr. C. Hidalgo. Stock solutions were 10 mM in ethanol. TEMPO was from a sample previously synthesised by one of us (M.J.P.) according to the method of Rozantsev (1970). NCD-4 and NCP were purchased from Molecular Probes Inc., Junction City, OR. All carbodiimide stock solutions were 5 mM in ethanol/dimethylformamide (1:1). Unless specified, all other chemicals and reagents were obtained from Sigma Chemical Co.

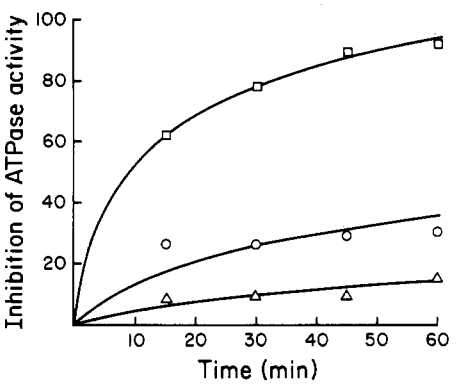


FIGURE 1: Effect of DCCD, NCD-4, and NCP on the ATPase activity of ETP_H. To 1 mL of ETP_H suspension was added 2 μL of an ethanolic solution (5 mM) of carbodiimide (5 nmol/mg of protein); the mixture was incubated on ice. Aliquots were removed for assay of ATPase activity according to Materials and Methods. DCCD (□); NCD-4 (○); NCP (△).

Results

The effects of NCD-4 and NCP on the ATPase activity of ETP_H are shown in Figure 1, where they are compared to the parent inhibitor DCCD. Under the conditions used, i.e., 5 nmol of reagent incubated with 1 mg of ETP_H on ice, enzyme activity is >90% inhibited by DCCD after 1 h, whereas the analogues NCD-4 and NCP exert a much smaller inhibition. It will be shown later that complete inhibition by NCD-4 and NCP can be achieved by longer incubation times although it is quite clear from Figure 1 that the order of reactivity is DCCD ≫ NCD-4 > NCP.

Solutions of NCD-4 or NCP in water or organic solvents are nonfluorescent. However, when NCD-4 (50 μM) was allowed to react with acetic acid (30 mM) in 95% ethanol for 1 h at 0 °C, a strongly fluorescent product was obtained. Uncorrected emission and excitation spectra for this product are shown in Figure 2A. The emission maximum of 434 nm is in good agreement with the data of Chadwick & Thomas (1983), who reported that the *N*-acetylurea from NCD-4 has an emission maximum of 425 nm in 100% ethanol and 440 nm in 50% ethanol (uncorrected spectra).

As with aqueous solutions, a suspension of ETP_H incubated with NCD-4 or NCP displays no immediate fluorescence. However, after incubating ETP_H for 24 h on ice with 12.5 nmol of reagent/mg of protein, followed by washing and resuspension, fluorescent products were again observed. Uncorrected emission spectra are shown in Figure 2B (upper spectra). In this case, emission maxima were at 398 nm for NCD-4 (spectrum on the left) and, because of vibrational fine structure, three maxima at 377, 387, and 396 nm for the pyrene derivative, NCP (spectrum on the right). Most importantly, Figure 2B shows that DCCD competes with the fluorescent analogues for the site of covalent attachment which is a prerequisite for the formation of a stable *N*-acylurea. Thus, treatment of ETP_H with DCCD at 5 nmol/mg protein for 30 min on ice followed by a 24-h incubation with NCD-4 or NCP and removal of unbound reagent as before gave rise to the lower spectra; i.e., the formation of a fluorescent conjugate was markedly inhibited in both cases.

Carbodiimide inhibition of ATPase activity may involve (i) noncovalent binding of the inhibitor, (ii) formation of an *O*-acylisourea intermediate, and (iii) rearrangement to a stable *N*-acylurea. The exact contribution of each step to the overall inhibition is not known. On the other hand, the generation of fluorescence by NCD-4 and NCP involves all three stages since it arises from the *N*-acylurea [Chadwick & Thomas (1983) have shown that the *O*-acyl intermediate of NCD-4

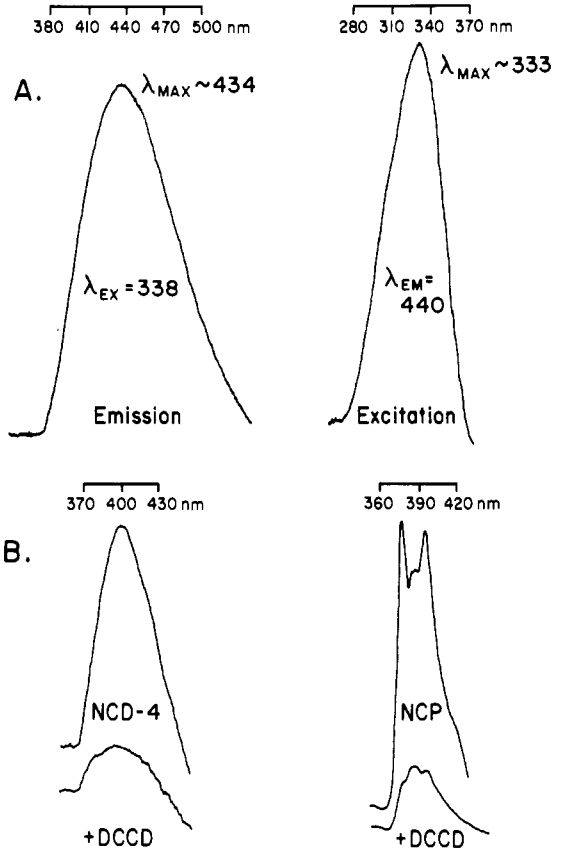


FIGURE 2: Fluorescence properties of NCD-4 and NCP. (A) Reaction between NCD-4 and acetic acid. To a solution of NCD-4 in 95% ethanol (50 μM) was added glacial acetic acid (30 mM); the mixture was incubated at 0 °C for 1 h. The excitation and emission spectra were recorded at 20 °C. (B) (Upper spectra) Five milliliters of ETP_H suspension was incubated with 25 μL of NCD-4 or NCP (5 mM) for 24 h at 0 °C, after which the samples were spun down and resuspended at 10 mg of protein/mL in fresh buffer. Aliquots (50 μL) were transferred to fluorescence cuvettes containing 1.95 mL of Tris/sucrose buffer, and fluorescence emission spectra were recorded at 20 °C. (Lower spectra) Samples of ETP_H similar to the above were incubated with 10 μL of DCCD (5 mM) for 30 min at 0 °C, after which 25 μL of NCD-4 or NCP (5 mM) was added and the samples were treated as before.

has an extremely low quantum yield compared to the *N*-acylurea]. We have, therefore, compared the time course for ATPase inhibition with that of the appearance of fluorescence under conditions in which both reagents were able to effectively inhibit enzyme activity. Thus, Figure 3A shows simultaneous kinetic profiles for the loss of ATPase activity (open circles) and the increase in fluorescence intensity (closed circles) for ETP_H incubated with NCD-4, where the fluorescence amplitudes have been normalized to that obtained at 24 h. Corresponding data for NCP are presented in Figure 3B (triangular symbols). In the latter case, it can be seen that the time courses for enzyme inhibition and fluorescence enhancement are similar (semilogarithmic plots give pseudo-first-order rate constants of 0.0016 and 0.002 min^{-1}, respectively), while for NCD-4 it would appear that ATPase inhibition is biphasic; i.e., the initial rapid decline in ATPase activity is not reflected in the fluorescence kinetics. On the other hand, the fluorescence profiles for both NCD-4 and NCP are remarkably similar.

In addition to inhibiting ATP hydrolysis, DCCD is a potent blocker of proton translocation through the mitochondrial H^+–ATPase complex. We have, therefore, examined the effects of all three carbodiimides on ATP-driven membrane potential by using the voltage-sensitive dye oxonol VI (Pringle

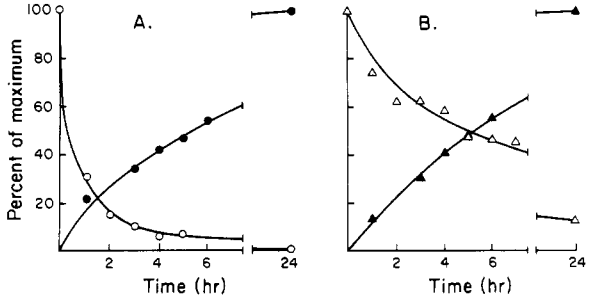


FIGURE 3: Kinetics of ATPase inhibition and fluorophore formation. Two milliliters of ETP_H was incubated on ice with 10 μL of NCD-4 (5 mM). Aliquots were removed periodically and assayed for ATPase activity as in Figure 1. For fluorescence measurements, 0.25-mL samples were removed and added to 1.75 mL of buffer. Emission spectra were scanned from 370 to 430 nm with an excitation wavelength of 338 nm. Emission amplitudes at 398 nm were normalized to the value obtained after 24-h incubation and plotted as percentages of this maximum value. In (A) the inhibitor was NCD-4. ATPase activity (○); fluorescence intensity (●). In (B) the inhibitor was NCP. ATPase activity (△); fluorescence intensity (▲).

& Sanadi, 1984). Figure 4A shows the time-dependent changes in oxonol VI absorbance for ETP_H energized with ATP/Mg^{2+} after incubation with either DCCD, NCD-4, NCP, or no inhibitor (traces a–d). The incubation conditions were 3 h at 0 °C with 12.5 nmol of inhibitor/mg of protein. Under these conditions, DCCD totally abolished the membrane potential, while NCD-4 and NCP inhibited the steady-state potential by 50% and 30%, respectively. A partial time course for the effect is shown in Figure 4B where it can be seen that DCCD exerted full inhibition after 1 h. For the fluorescent analogues, we were able to demonstrate total inhibition of ATP-driven membrane potential after a 24-h incubation with the inhibitors (data not shown). On the other hand, energization with NADH was not affected by treatment of ETP_H with the fluorescent carbodiimides. A typical dye response to NADH after NCP treatment is shown in Figure 4C. NCD-4 was similarly without effect (data not shown).

In order to obtain information on (a) the accessibility of the fluorescent probe binding site to the lipid bilayer and (b) the location of this binding site with respect to its distance from the membrane surface, we conducted paramagnetic fluorescence quenching experiments on purified F_1–F_0 vesicles extracted with lysolecithin from ETP_H (Hughes et al., 1982) and subsequently incubated with NCD-4 or NCP at a ratio of 6 mol of inhibitor/mol of F_1–F_0. After the vesicles were labeled, the ATPase activity had dropped from 1.99 units to 0.05 (NCD-4) and 0.09 unit (NCP). As quenchers, we employed a series of stearic acid spin-labels denoted *x*-NS (where *x* indicates the carbon atom on which the paramagnetic nitroxide group is located) and the membrane-permeable spin-label TEMPO. The stearic acid spin-labels have been used for many years as probes of the "fluidity" gradient of lipid membranes in a direction normal to the membrane plane (Hubbell & McConnell, 1969, 1971). The usefulness of these reagents stems from the fact that the carboxyl group when ionized essentially anchors the molecule at the membrane surface and allows the hydrocarbon chain to intercalate with the phospholipid acyl chains.

Dynamic collisional, or Stern–Volmer, quenching obeys the relationship:

$$I_0/I = 1 + k_q\tau_0[Q] = 1 + K_D[Q] \quad (1)$$

where I_0 and I are the fluorescence intensities in the absence and presence of the quencher, k_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence

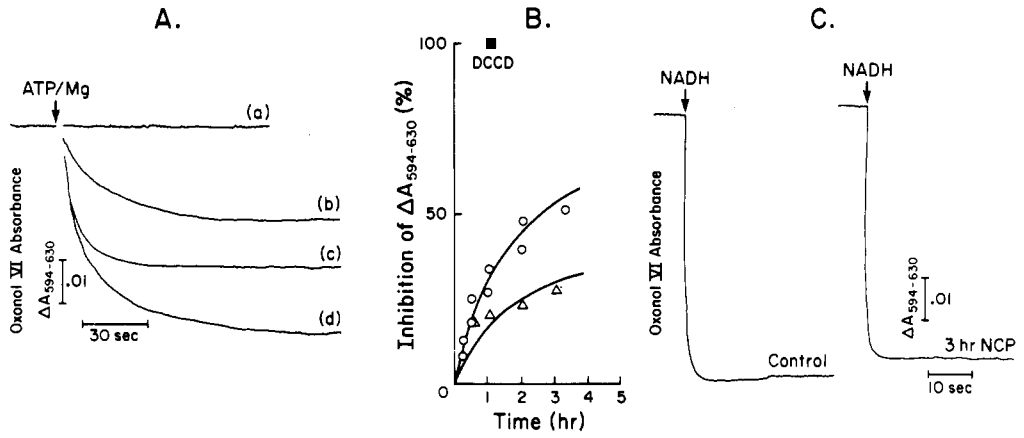


FIGURE 4: Effects of DCCD, NCD-4, and NCP on proton translocation in ETP_H. One milliliter of ETP_H suspension was incubated on ice with 5 μL of carbodiimide inhibitor. (A) shows spectrophotometric traces of changes in oxonol VI absorbance ($\Delta A_{594-630}$) accompanying energization (20 μL of 5 mM ATP/Mg^{2+}) of 50-μL aliquots of inhibitor-treated ETP_H vesicles after a 3-h incubation. (a) DCCD; (b) NCD-4; (c) NCP; (d) control. (B) shows the kinetics of inhibition of steady-state membrane potential ($\Delta A_{594-630}$) under the experimental conditions specified above. NCD-4 (○); NCP (△); effect of DCCD after 1 h is shown for comparison (■). (C) shows the lack of effect of NCP on the oxonol VI absorbance change of ETP_H energized with NADH. The conditions were as in (A) except that the generation of the membrane potential was initiated by adding 10 μL of 30 mM NADH.

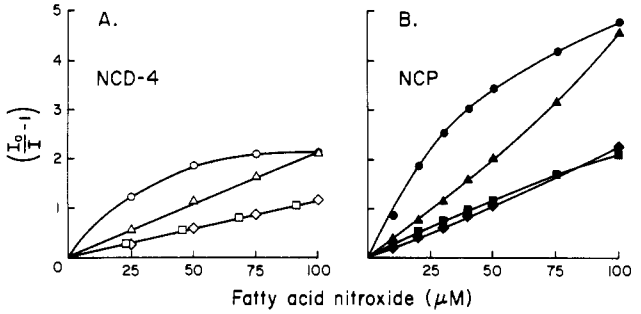


FIGURE 5: Quenching of NCD-4 and NCP fluorescence with stearic acid nitroxides. Two milliliters of purified H^+–ATPase suspension (containing 1 mM dithiothreitol) was incubated with 10 μL of either NCD-4 or NCP (5 mM) for 72 h at 0–4 °C, after which the labeled vesicles were diluted 3.5-fold, spun down, and resuspended to 2 mg of protein/mL in fresh buffer. For fluorescence measurements, 0.25 mL of the labeled suspension was added to 1.75 mL of buffer and the excitation wavelength set at 338 nm. Fluorescence emission was recorded at 400 (NCD-4) and 398 nm (NCP). Stearic acid nitroxides (10 mM stock solutions) were added directly to the assay cuvettes, and after allowing for equilibration (2–5 min), the new fluorescence intensity was recorded. For a definition of the Stern–Volmer parameter ($I_0/I - 1$), see eq 1 under Results. For both (A) and (B), the symbols are 5-NS (diamonds), 7-NS (squares), 12-NS (triangles), and 16-NS (circles).

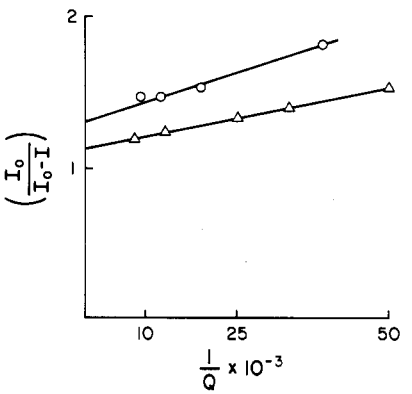


FIGURE 6: Population of fluorophores inaccessible to quenching by 16-NS. The quenching data for 16-NS (Figure 5, open and closed circles) were replotted according to eq 2 given under Results. The inaccessible fraction of fluorophores is $1 - f_a$, where f_a^{-1} is the intercept on the ordinate. NCD-4 (○); NCP (△).

of quencher, [Q] is the concentration of quencher, and K_D is the Stern–Volmer quenching constant. Thus, a Stern–Volmer plot of $I_0/I - 1$ vs. [Q] should yield a straight line whose slope is equal to the dynamic quenching constant K_D. If, however, a fraction of fluorophores is inaccessible to the quencher, the Stern–Volmer plot is downward curving to the concentration axis, and the following relationship obtains (Lehrer, 1971; Lehrer & Leavis, 1978):

$$I_0/(I_0 - I) = 1/f_aK[Q] + 1/f_a \quad (2)$$

where K is the quenching constant of the accessible fraction and f_a is the fraction of initial fluorescence accessible to the quencher. Thus, a plot of $I_0/\Delta I$ vs. $1/[Q]$ will be a straight line of intercept f_a^{-1} and slope $(f_aK)^{-1}$.

Figure 5 shows the quenching effects of the stearic acid spin-labels 5-NS, 7-NS, 12-NS, and 16-NS on the fluorescence of F_1–F_0 vesicles labeled with NCD-4 (Figure 5A) and NCP (Figure 5B). The Stern–Volmer plots for both fluorophores show that at low quencher concentrations, the order of quenching efficiency is 16-NS > 12-NS > 7-NS ≃ 5-NS; i.e.,

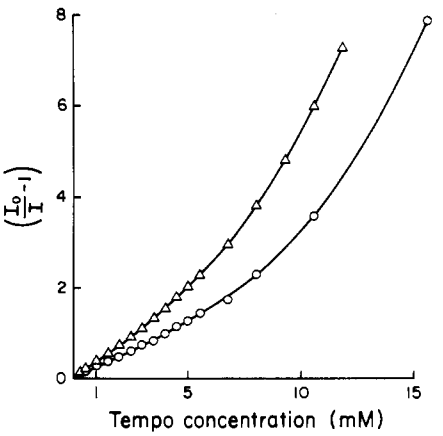


FIGURE 7: Fluorescence quenching of labeled H^+–ATPase by the spin probe TEMPO. The experimental details of inhibitor labeling and fluorescence measurements were the same as in Figure 5. TEMPO stock solutions were 100 or 500 mM in water; NCD-4 (○); NCP (△).

the effect is maximal when the paramagnetic group is deepest in the bilayer. However, it can also be seen in Figure 5 that, at higher concentrations, the concentration dependence of quenching by 16-NS (the most efficient quencher) is downward curving. The data for 16-NS were, therefore, replotted according to eq 2 and afforded straight lines (Figure 6) from

which the fraction of accessible fluorophores, f_a, was calculated to be 88% for NCD-4 and 77% for NCP and, hence, the inaccessible fractions were 12% and 23%, respectively. The freely permeant spin-label TEMPO was also able to effectively quench both NCD-4 and NCP fluorescence (Figure 7), whereas iodide ions (highly efficient quenchers) exerted almost negligible effect on the fluorescence of either NCD-4 or NCP (data not shown).

Discussion

Enzyme Inhibition. NCD-4 and NCP are analogues of DCCD in which one of the cyclohexyl rings has been replaced by *p*-(dimethylamino)-α-naphthalene (NCD-4) or pyrene (NCP). DCCD itself is a highly potent and specific inhibitor of the mitochondrial H^+–ATPase. Its mode of action involves covalent modification of a glutamic acid residue on the 8000-dalton proteolipid subunit of the F_0 portion of the enzyme complex (Sebald & Wachter, 1978, 1980). We have shown here that NCD-4 and NCP are also effective inhibitors of ATPase activity and ATP- (but not NADH-) driven proton translocation in bovine mitochondrial ETP_H. The latter would suggest that, in our experiments, the carbodiimides react specifically with the H^+–ATPase and not with other components of the respiratory chain such as cytochrome oxidase.

It is worth commenting on the different efficacies of all three carbodiimides with respect to inhibition of ATPase activity and ATP-driven membrane potential. It is clear from Figure 1 that DCCD reacts faster than either NCD-4 or NCP in inhibiting ATPase activity. In fact, one can calculate from the data in Figures 1 (DCCD) and 3 (NCD-4 and NCP) that the pseudo-first-order rate constants for inhibition are 0.037, 0.008, and 0.0016 min^{-1} for DCCD, NCD-4, and NCP, respectively. Inhibition of the steady-state ATP-driven membrane potential, on the other hand, would appear to be a slower process; i.e., for NCD-4, k_1(ATPase) = 0.008 and k_1-(steady-state potential) = 0.005 min^{-1} (data from Figure 4B). This is in contrast to the assertion of Kiehl & Hatefi (1980) that, in complex V, ATP–P_i exchange is more susceptible to DCCD inhibition than to ATPase activity. The discrepancy may well arise from the fact that although treatment with inhibitor is carried out at 0 °C, our ATPase assay requires a 10-min incubation at 30 °C with, presumably, inhibitor still bound to the vesicle membrane. In the case of Kiehl & Hatefi (1980) and Stiggall et al. (1978), the converse was true; i.e., their ATP–P_i exchange assay required a 5-min incubation at 30 °C, while their ATPase assay was time-resolved. In fact, it has been shown in this laboratory that when the assay conditions are made similar, there is no kinetic difference between ATPase inhibition and ATP–P_i exchange inhibition (Hughes et al., 1982).

Fluorescence Properties. Neither NCD-4 nor NCP exhibits fluorescence in solution or in the immediate presence of membrane vesicles. However, both reagents form highly fluorescent products when allowed to react for several hours with mitochondrial ETP_H or the purified H^+–ATPase (F_1–F_0) (see legend to Figure 5). The fluorescence properties of NCD-4 in sarcoplasmic reticulum vesicles have already been described by Chadwick & Thomas (1983), who reported maximum emission wavelengths for the *N*-acetylurea of NCD-4 in hexane (398 nm), ethanol (425 nm), and ethanol/water (1:1) (440 nm). This would suggest that in ETP_H and F_1–F_0 vesicles (λ_{max} = 398 nm), the environment of the probe binding site is extremely hydrophobic. We have shown (Figure 2B) that the fluorescence of NCD-4 and NCP is inhibited by prior treatment of ETP_H with DCCD which is good evidence that the fluorescent analogues and DCCD compete for the same binding site.

Fluorescence Quenching. Nitroxide radicals were shown many years ago to quench the fluorescence of aqueous solutions containing fluorophores (Singer & Davis, 1967; Buchachenko et al., 1967). Since then, several studies have been carried out in biomembranes or lipid bilayer systems where nitroxide spin-labels have been used to quench intrinsic protein fluorescence (Wallach et al., 1974; Bieri & Wallach, 1975, 1976) or that due to noncovalently bound probe molecules (Luisetti et al., 1979). In the latter study, stearic acid spin-labels were successfully used to quench the fluorescence (and thereby probe the fluorophore location) of pyrenebutyric acid and pyrenedecanoic acid in lipid bilayers. The ranking order of quenching efficiency for the spin-labels correlated well with the respective distances between the pyrene moiety and the carboxyl group. The spatial resolution of the method would seem to be limited by an interaction distance of 4–6 Å between quencher and fluorophore (Green et al., 1973).

In the present report, we show that stearic acid spin-labels can quench the fluorescence of extrinsic fluorophores covalently attached to membrane proteins. The fact that quenching of NCD-4 or NCP fluorescence by interaction with fatty acid derivatives occurs at all indicates that the fluorophore binding site, which we presume to be glutamic acid residue 61 of the proteolipid (Sebald & Wachter, 1980), is located in the lipid bilayer and accessible to the membrane phospholipids rather than in a hydrophilic cavity of the protein. The hydrophobic nature of the binding site is borne out by the maximum emission wavelength for NCD-4 or NCP fluorescence, i.e., <400 nm in both cases. Chadwick & Thomas (1983) report the maximum emission wavelength of the *N*-acetyl derivative of NCD-4 as being 398 nm in hexane and 425 nm in ethanol. Further qualitative support for the preceding derives from the fact that the spin-label TEMPO, which is both water soluble and freely membrane permeable, is a reasonably effective quencher; i.e., at 10 mM, NCD-4 and NCP fluorescence are quenched by 75% and 85%, respectively (calculated from the data in Figure 7).

The second important finding of the present study is that, for both NCD-4 and NCP bound to F_1–F_0 vesicles, the degree of fluorescence quenching by fatty acid spin-labels correlates well with the depth of the paramagnetic moiety within the bilayer. Nonpermeant iodide ions (highly efficient quenchers) had negligible effect on the fluorescence of either reagent. Thus, 16-NS was the most effective and 5-NS the least effective quencher among the group of stearic acid spin-labels used (Figure 5A,B). The differential sensitivity to quenching is most apparent at low quencher concentration (<50 μM), and we would place more reliability on this concentration range since, even at 25 μM, the fatty acid nitroxides represent ca. 10 mol % of the total membrane phospholipid (250 μM in the quenching assays based on a measured protein/lipid ratio of 1.3 w/w). This would put the fluorophore binding site at ~18 Å from the membrane surface. We cannot, of course, exclude the possibility that 14-NS (untested in this study) might be an even more effective quencher than 16-NS. Furthermore, we must also acknowledge that the greater degree of lipid acyl chain mobility in the bilayer center, compared to the region near the membrane surface, may contribute to the enhanced quenching by nitroxide groups located deeper in the bilayer. However, given the above caveats, and the uncertainty intrinsic to an interaction distance of 4–6 Å, our results provide reasonable corroboration for the recent suggestion by Senior (1983), based on hydropathy plots, that in *E. coli* the DCCD binding site is located 15 Å from the membrane surface.

Finally, the quenching behavior of 16-NS (Figure 5A,B) deserves some comment. As we have stated earlier, a downard curving Stern–Volmer plot is obtained when a population of fluorophores is inaccessible to the quencher molecules. We should also point out that this type of saturation behavior was exhibited by all the stearic acid nitroxides tested, though in the case of 5-, 7-, and 12-NS it occurred at considerably higher concentrations than the range shown in Figure 5. At comparable levels of fluorescence quenching (magnitude of the Stern–Volmer parameter) the spin-label TEMPO did not exhibit saturation-type behavior (Figure 7). From Figure 6, the fraction of inaccessible fluorophores was calculated to be 23% and 12% for NCD-4 and NCP, respectively. Since the calculation requires a back extrapolation of only the linear portion of the plots in Figure 6 (Lehrer & Leavis, 1978), our limited data are probably inadequate to distinguish between the two values. Taking a mean value, then, the inaccessible fraction is about 18% or one-sixth of the total fluorophore population. We have tried to visualize the labeled sites on sodium dodecyl sulfate gels but have been unable to observe any fluorescence bands, since the emission from NCD-4 or NCP conjugates is at the UV/visible wavelength border (400 nm). The interpretation of our data, therefore, is based on the assumption that only the proteolipids are labeled. Furthermore, since we have used low levels of carbodiimide in order not to label sites other than the proteolipid, it is even unlikely that all six copies of the subunit would be labeled. Thus, although only one-sixth of the stoichiometric amount of DCCD is required to totally inactivate the H^+-ATPase (Sebald et al., 1976; Graf & Sebald, 1978), we have no unequivocal evidence that the inaccessible fraction of fluorophores represents a structurally different DCCD binding site on one of the proteolipids.

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Registry No. NCD-4, 86332-16-3; NCP, 98540-87-5; DCCD, 538-75-0; ATPase, 9000-83-3; 5-NS, 29545-48-0; 7-NS, 40951-82-4; 12-NS, 29545-47-9; 16-NS, 53034-38-1; TEMPO, 2564-83-2; H^+, 12408-02-5.

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