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INACTIVATION OF SARCOPLASMIC RETICULUM (Ca^{2+} + Mg^{2+})-ATPase BY *N*-CYCLOHEXYL-*N'*-(4-DIMETHYLAMINO-α-NAPHTHYL)CARBODIIMIDE

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The synthesis and characterisation of *N*-cyclohexyl-*N'*-(4-dimethylamino-α-naphthyl)carbodiimide (NCD-4) is described. Only the *N*-acetylurea and urea corresponding to NCD-4 are appreciably fluorescent: the *O*-phenylisourea and *S*-ethylisothiourea derivatives have negligible fluorescence. NCD-4 inhibits the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum irreversibly: Ca^{2+} protects against inhibition. Covalent incorporation of NCD-4 occurs into the Ca^{2+}-protected sites, with a stoichiometry of approximately 1 mole/mole of ATPase. The modified enzyme has fluorescence emission properties similar to those of NCD-4 *N*-acetylurea in a relatively hydrophobic environment: it is concluded that NCD-4 has modified a carboxylate group (s) located in or near the Ca^{2+}-binding sites of the ATPase.

Introduction

Dicyclohexylcarbodiimide (DCCD) is an irreversible inhibitor of a number of energy-transducing systems, including various ATPases [1–3], cytochrome oxidase [4], transhydrogenase [5], and cytochrome bc_1 [6]. In order to exploit this labelling capacity for studies on these systems by fluorescence techniques, we have synthesized the title compound *N*-cyclohexyl-*N'*-(4-dimethylamino-α-naphthyl)carbodiimide (NCD-4). NCD-4 is itself nonfluorescent, but its *N*-acylurea, a likely reaction product with aspartic acid and glutamic acid side-chains, is strongly fluorescent. The preparation and characterisation of NCD-4, together with details of its irreversible inhibition of the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum, is now described.

Materials and Methods

Synthesis of NCD-4. Equimolar amounts of freshly distilled cyclohexylamine and 4-dimethylaminonaphthalene-1-isothiocyanate (Sigma) were incubated in diethyl ether for 12 h at 23°C. *N*-Cyclohexyl-*N'*-(4-dimethylamino-α-naphthyl)thiourea, m.p. 164–166°C, precipitated from the reaction mixture, being obtained in 90% yield after filtration and washing with ice-cold ether.

Treatment of the thiourea (0.5 g) in dry acetone (20 ml) with yellow mercuric oxide (1 g) at 80°C for 3 h resulted in complete conversion to NCD-4. The reaction mixture was then cooled, filtered through Celite, and evaporated to dryness. Column chromatography over silica gel (Merck, Kieselgel 60) using chloroform/hexane (1 : 1, v/v) as eluant then gave pure NCD-4, obtained as a pale yellow oil on evaporation of appropriate eluate

Abbreviations: Mops, 3-(*N*-morpholino)propanesulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; NCD-4, *N*-cyclohexyl-*N'*-(4-dimethylamino-α-naphthyl)carbodiimide; DCCD, dicyclohexylcarbodiimide.

fractions. The sample proved homogenous on TLC examination in a range of solvent systems, displayed a strong infrared band at 2100 cm^{-1} (N = C = N stretch), and had λ_{max} 335 nm, $\epsilon = 10.25 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ (ethanol).

NCD-4 proved stable in ethanolic solution at 0°C for several weeks. Partly decomposed samples were readily purified by column chromatography as above.

Synthesis of NCD-4 acetylurea and NCD-4 urea. Treatment of NCD-4 (200 mg) in methylene-chloride (5 ml) with glacial acetic acid (0.5 ml) for 2 h at 23°C gave complete conversion to two fluorescent products, as shown by TLC. Column chromatography over silica gel using chloroform as eluant gave firstly the *N*-acetylurea: m.p. 119–120°C; ν_{max} 1660, 1700 cm^{-1}; λ_{max} 325 nm; $\epsilon = 5.7 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ (ethanol). The 90 MHz ^{1}H-NMR spectrum (C^2HCl_3) showed diagnostic signals at δ 1.85 (3H, $-NCOCH_3$), δ 2.8 (6H, $N(CH)_3)_2$), δ 3.2 (s, 1H, exch ^2H_2O, NH). A low-resolution mass spectrum did not show the expected molecular ion at M^+ 353, but a peak at M^+ 228 was observed. Production of a M^+ 228 fragment can be rationalised in terms of a facile McLafferty cleavage of the *N*-acetylurea, provided that the *N*-acetyl function is placed on the nitrogen atom proximal to the napthalene ring.

NCD-4 urea, obtained by continued elution with chloroform, had m.p. 185°C, ν_{max} 1630 cm^{-1}, λ_{max} 325 nm, $\epsilon = 4.7 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ (ethanol), M^+ (low resolution) 311.

Synthesis of NCD-4-O-phenylisourea (cf. Ref. 7). NCD-4 (250 mg) and phenol (200 mg) were heated for 12 h at 100°C under nitrogen. After cooling and dilution with diethyl ether, excess phenol was removed by extraction with 1 M aqueous NaOH. The *O*-phenylisourea (250 mg) was then isolated by column chromatography and crystallised from acetone/water, m.p. 85°C. It had λ_{max} 330 nm, $\epsilon = 6.8 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ (ethanol). The 90 MHz ^{1}H-NMR spectrum (C^2HCl_3) showed diagnostic signals δ 1.8–3.3 (multiplets, 11 H, aromatic ring protons), δ 4.0 (s, 1H, exch ^2H_2O, NH) and δ 7.2 (s, 6H, $N(CH_3)_2$).

Synthesis of NCD-4-S-ethylisothiourea. NCD-4 on treatment with excess ethanethiol containing 1 drop Et_3N was rapidly converted to the isothiourea, isolated by column chromatography. The material, obtained as a pale green oil, had λ_{max} 335 nm, $\epsilon = 8.9 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ (ethanol). The 60 MHz ^{1}H-NMR spectrum ($CHCl_3$) showed diagnostic signals at δ 1.5–3.5 (multiplets, 6H, aromatic ring), 6.4 (s, exch ^2H_2O, 1H, NH), 7.2 (s, 6H, $N(CH_3)_2$; quartet, 2H, methylene, SEt group).

Synthesis of ^{3}H-labelled NCD-4. (2,2,6,6-^{3}H)-labelled cyclohexylamine was prepared by (i) exchange titration of cyclohexanone (cf. Ref. 8), followed by (ii) conversion to cyclohexanone oxime and finally (iii) reduction to cyclohexylamine. Freshly distilled cylchohexanone (0.5 g), tritiated water (1 ml, 0.5 mCi) and potassium carbonate (50 mg) were incubated at 120°C for 48 h with stirring in a sealed system. The cooled reaction mixture was then treated with an aqueous solution of hydroxylamine hydrochloride (800 mg) preadjusted to pH 7, and the mixture heated to 80°C for 30 min. Cyclohexanone oxime was then isolated by extraction into pentane; the pentane extracts were combined, washed with water, and evaporated. The crystalline oxime (510 mg) was dried over P_2O_5 in vacuo before use.

To a stirred suspension of lithium aluminium hydride (120 mg) in dry tetrahydrofuran (2 ml), ^{3}H-labelled cyclohexanone oxime (120 mg) was added at 0°C under nitrogen. The reaction mixture was then allowed to reach 23°C, and was then refluxed for 15 min. Careful dropwise addition of 20% (w/v) aqueous sodium hydroxide solution to the stirred and cooled reaction mixture gave an insoluble gel. The supernatant, together with appropriate washings of the gel, was decanted directly onto solid 4-dimethylaminonaphthyl-1-isothiocyanate (200 mg). After brief refluxing, this reaction mixture was diluted with diethyl ether, washed with water, dried (Na_2SO_4), and evaporated in vacuo to give the crude thiourea. Column chromatography over silica gel gave pure thiourea, 130 mg, m.p. 166°C. Conversion to [^{3}H]NCD-4 was effected as described above: the final product proved chemically and radiochemically pure, having a specific activity of 33 $cpm \cdot nmol^{-1}$ as assayed under the conditions described below.

Reactivities of NCD-4 and dicyclohexylcarbodiimide [9]. Reaction rates with acetic acid were measured in tetrahydrofuran at 23°C. Reaction mixtures containing the appropriate carbodiimide

and acetic acid at final concentrations of 47 mM and 1 M respectively were made up, rapidly transferred into 0.1 mM pathlength infrared cells, and the disappearance of the 2100 cm^{-1} band monitored using a Unicam S.P. 2000 spectrophotometer. Transmission values were converted to the corresponding absorbance at first order reaction rates derived from a plot of log $A_t - A_\infty$ versus time.

Preparation and characterisation of sarcoplasmic reticulum vesicles. Vesicles were isolated from rabbit skeletal muscle essentially as described earlier [10]. All extraction buffers contained 1 mM dithiothreitol and 5 μM phenylmethylsulfonyl fluoride. Vesicles were routinely washed [11] by overnight incubation in 0.6 M KCl followed by two washes in 0.3 M sucrose/0.6 M KCl/10 mM Mops (pH 7.0) and final resuspension (10 mg · ml^{-1}) in 0.3 M sucrose/5 mM Mops (pH 7.0). Vesicles were stored in liquid nitrogen for up to 3 months prior to use.

Ca^{2+}, Mg^{2+}-dependent ATPase activities were measured at 37°C as described [12]: the assay medium contained the ionophore A23187 (5 μg · ml^{-1}). Preparations typically had Ca^{2+}, Mg^{2+}-dependent ATPase activities of 12–14 μmol · min^{-1} · mg^{-1} of vesicular protein. Protein content was measured by the method of Lowry et al. [13] using bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis of the preparations [10,14] followed by densitometric scanning of the Coomassie blue stained gels showed that the ATPase component made up approx. 75% of the total protein content. The preparations had a thiol content of 13–15 mol/10^5 g of protein as measured in 1% SDS. Phospholipid content [15] using dipalmitoylphosphatidylcholine (Sigma) as standard was typically 0.6 μmol · mg^{-1} vesicular protein.

Inhibition of Ca^{2+}, Mg^{2+}-dependent ATPase activity by NCD-4 and DCCD. Sarcoplasmic reticulum vesicles (1 mg · ml^{-1} protein) were treated at 23°C in 100 mM KCl/50 mM Mes (pH 6.2)/1 mM EGTA, without or with 1.25 mM $CaCl_2$ with the appropriate carbodiimide (150 μM) added from an ethanol stock solution (15 mM). Aliquots were removed at appropriate time intervals and assayed as above.

Stoichiometry of NCD-4 incorporation. Incubation with ^{3}H-labelled NCD-4 was performed as described above (reaction volume = 10 ml). After 3 h the reaction was quenched by addition of 1.0 ml ice-cold 500 mM Mops buffer (pH 7.9), followed by 20 ml ice-cold 0.3 M sucrose/5 mM Mopes (pH 7.0). Vesicles were collected by centrifugation at 4°C (80000 × g, 90 min), and resuspended in 1.0 ml 0.3 M sucrose/5 mM Mops (pH 7.0). The resultant suspension was injected into 15 ml of ice-cold chloroform/methanol (2 : 1, v/v) with vortexing, and the precipitated protein collected by filtration on fibre-glass disks. The precipitates were washed with 6 × 10 ml volumes of chloroform/methanol, and air dried for 2 h prior to solubilisation in 1% (w/v) sodium dodecyl sulphate at 80°C for 60 min. Suspensions were then clarified by centrifugation and Millipore filtration, and suitable aliquots assayed for radioactivity. Protein content was determined spectrophotometrically assuming an A_{280} of 1.0 for a 1 mg · ml^{-1} solution of vesicular protein in 1% SDS [16]. Liquid scintillation counting was performed using a Triton X-100 cocktail [17].

Localisation of fluorescent label in vesicular proteins. SDS-polyacrylamide gels of vesicles which has been labelled in the presence or absence of calcium were prepared as described [14]. Gels were then fixed in ethanol/acetic acid, and scanned for fluorescence using 338 nm excitation and 430 nm emission wavelengths. A home-built scanner interfaced with a Perkin-Elmer 1000 spectrofluorimeter was used. Tryptic digests of labelled vesicles were also prepared [16] and samples electrophoresed and scanned for fluorescence as above. After fluorescence scanning, gels were stained (Coomassie blue) and scanned for absorbance.

Fluorescence methods. Uncorrected spectra were obtained using a Schoeffel RRS 1000 spectrofluorimeter interfaced to a Apple II-plus microcomputer through a Synertek 6522 V.I.A. which was wired to an ADC 0817 CCN analogue-to-digital converter (Radiospares Ltd.). Plots of fluorescence intensity as a function of wavelength were made using the high-resolution graphics capability of the microcomputer. Data were stored on floppy disc and plotted on a T.C.M. thermal printer when hard copy was required. Corrected emission spectra were obtained using a home-built fully corrected instrument [18]. All other measurements were made using a Perkin-Elmer 1000 spectrofluorimeter.

Quantum yields were determined as described [19,20] using quinine sulphate (Sigma) in 1.0 N H_2SO_4 as standard, assuming $\phi = 0.546$ at 25°C, excitation 330 nm.

Results and Discussion

Fluorescence properties of NCD-4 and its adducts

NCD-4 proved non-fluorescent when examined in a range of solvents at room temperature. However, the *N*-acetylurea, and particularly the corresponding urea, were strongly fluorescent (Table I). The emission maxima of both the urea and *N*-acetylurea respond to changes in solvent polarity (Table I). In contrast, the *S*-ethylisothiourea and *O*-phenylisourea, formed by reaction with ethanethiol and phenol, respectively, had very low fluorescence quantum yields: the *O*-phenylisourea having an estimated quantum yield of 0.003 (ethanol), while the *S*-ethylisothiourea showed no measurable fluorescence at 23°C in a range of solvents. Thus while modification of glutamic acid, aspartic acid, cystine and tyrosine residues by NCD-4 are possible, any fluorescence emission observed as a result of reaction will most likely originate from either protein-bound *N*-acylurea, or from urea formed by intracellular reaction of *O*-acylisourea [2] with suitably positioned protein nucleophilic groups.

TABLE I

UNCORRECTED FLUORESCENCE EMISSION MAXIMA OF NCD-4 DERIVATIVES IN VARIOUS SOLVENTS AT 23°C

NCD-4 urea proved too insoluble in hexane for spectral measurements. Excitation was at 330 nm. Values in parentheses represent corrected emission maxima.

NCD-4 derivative	λ_{em}(nm)		
	Hexane	Ethanol	Ethanol/H_2O (1:1, v/v)
N-Acetylurea	398	425 (430) $\phi = 0.06$	440
Urea	–	460 (467) $\phi = 0.58$	473
O-Phenylisourea	435	452	465

NCD-4 is less reactive than DCCD

The second order rate constants describing the reaction of the carbodiimides with acetic acid in tetrahydrofuran (23°C) indicate that NCD-4 (k_2 $10.6 \cdot 10^{-3}$ $M^{-1} \cdot min^{-1}$) is considerably less reactive than DCCD (k_2 $152 \cdot 10^{-3}$ $M^{-1} \cdot min^{-1}$).

NCD-4 irreversibly inhibits sarcoplasmic reticulum ($Ca^{2+} + Mg^{2+}$)-ATPase

Fig. 1 shows that in the absence of Ca^{2+}, NCD-4 at 150 μM inhibits the ATPase in a time-dependent manner: inclusion of Ca^{2+} (250 μM) gave complete protection. The second-order rate constants for inhibition of ATPase activity by the carbodiimides (23°C) indicate that NCD-4 ($k_2 =$ 130 $M^{-1} \cdot min^{-1}$) is somewhat less effective than DCCD ($k_2 = 430$ $M^{-1} \cdot min^{-1}$). Comparison of the reaction rates of the two carbodiimides with the ATPase and acetic acid (k_2(ATPase)NCD-4/DCCD = 0.31, k_2(acetic acid NCD-4/DCCD = 0.07) indicate that NCD-4 has a greater reactivity toward the ATPase than would be expected from its chemical reactivity. This may be a reflection of the greater hydrophobicity of NCD-4, supporting the view that the site at which carbodiimides attack, causing inhibition of ATPase activity, is situated in a hydrophobic environment.

Inhibition is accompanied by covalent incorporation

The fluorescence emission spectra of vesicles labelled with NCD-4 in the presence and absence of Ca^{2+} have emission maxima at 425–430 nm,

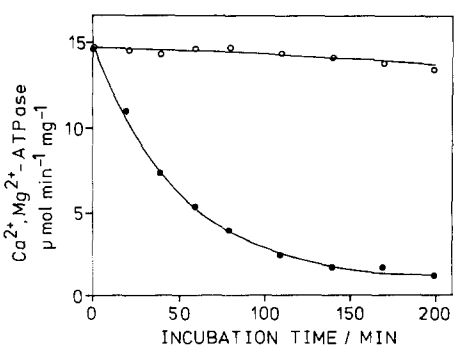

Fig. 1. Protection of ($Ca^{2+} + Mg^{2+}$)-ATPase activity by Ca^{2+} against irreversible inhibition by NCD-4. Conditions were as described in Materials and Methods. ○, incubation in the presence of Ca^{2+} (250 μM free); ●, incubation in the absence of Ca^{2+} (1 mM EGTA included).

which are indicative of *N*-acylurea in an 'ethanol' type environment (Table I). These spectra are completely unlike those of the urea in any solvent system studied. Deliberate incubation of vesicles with NCD-4 urea (added as small aliquots from and ethanol stock) resulted in its facile uptake, the emission maximum being at 460 nm. Accordingly, fluorescence due to reaction of NCD-4 most likely originates from protein bound *N*-acylurea, probably in a relatively hydrophobic environment. A time course of the fluorescence increase due to reaction in the presence and absence of Ca^{2+} is shown in Fig. 2: the difference in the curves shows saturation at a time corresponding to that at which maximal inactivation of ATPase activity is reached. Thus the fluorescence difference is attributed to reaction at a specific class of carboxyl groups which also results in inactivation of (Ca^{2+} + Mg^{2+})-ATPase activity. Fluorescence scans of SDS-polyacrylamide gels of vesicles labelled in the presence and absence of Ca^{2+} (Fig. 3a) indicated that the (Ca^{2+} + Mg^{2+})-ATPase was the only protein significantly labelled. Similar fluorescence scans of gels of tryptic digests prepared from NCD-4-labelled vesicles showed that the specific site is probably located in the 24 kDa fragment A_2 [21] (Fig. 3c): vesicles labelled in the absence of Ca^{2+} showed considerably greater fluorescence incorporation into this fragment relative to vesicles incubated in the presence of Ca^{2+}.

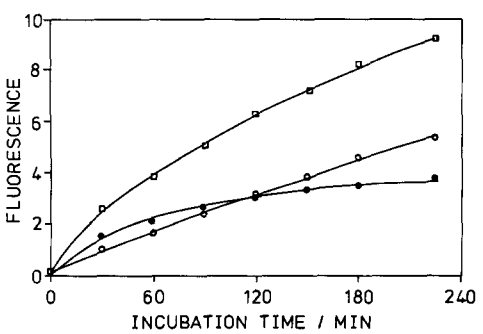

Fig. 2. Effect of Ca^{2+} inclusion on the final fluorescence intensity observed as a result of incubation of vesicles with NCD-4. Incubation conditions were as described. Fluorescence intensity (430 nm) was determined after 26-fold dilution of the reaction medium into 100 mM KCl/25 mM Mops (pH 7.0)/1.0 mM EGTA. Excitation was at 338 nm. O, incubation in the presence of Ca^{2+}; □, incubation in the absence of Ca^{2+}; ●, Ca^{2+}-dependent difference in fluorescence intensity.

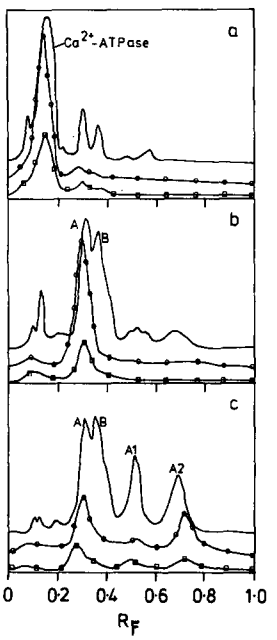

Fig. 3. Absorbance and fluorescence scans of SDS gels of vesicles which had been incubated with NCD-4 in the presence of Ca^{2+}. (a) No tryptic digestion. (b) 30 s tryptic digestion (c) 40 min tryptic digestion. Tryptic digestion was at 30°C in 25 mM Mops-Mes (pH 7.0)/60 mM KCl/1 M sucrose/4 mM $CaCl_2$/0.5 mg·ml^{-1} vesicular protein/50 μg·ml^{-1} trypsin. Digestions were quenched by addition of soyabean trypsin inhibitor (4 mg·mg^{-1} trypsin). After electrophoresis, gels were fixed and scanned for fluorescence (excitation 338 nm, emission 430 nm) and then stained and scanned for Coomassie blue absorbance. ———, absorbance; O———O, fluorescence of vesicles incubated in the absence of Ca^{2+}; □———□, fluorescence of vesicles incubated in the presence of Ca^{2+}.

Direct estimates of the extent of covalent incorporation of NCD-4 were made using the ^3H-labelled analogue. In the presence of Ca^{2+}, 5.6 nmol of NCD-4 are incorporated per mg vesicular protein. In the absence of Ca^{2+}, incorporation levels increase to 12.6 nmol per mg protein (the values are averages of three independent determinations). The difference in these values, approx. 7 nmol/mg protein, is attributed to labelling of the ATPase at sites which are protected by Ca^{2+}. Given that the ATPase (M = 115000) constitutes approx. 75% of the total vesicular protein in our preparations, approx. 6.5 nmol of ATPase per mg of total protein is indicated, leading to a stoichiometry of NCD-4 incorporation of approx. 1 mol/mol ATPase. This value may well be an

underestimate when levels of active ATPase in the preparation are taken into account: measurements of steady-state levels of the phosphorylated intermediate generally indicate values of 2–5 nmol/mg protein. In this light the stoichiometry of NCD-4 incorporation into Ca^{2+}-protected sites would then approach approx. 2 mol/mol active ATPase, which is equivalent to the number of high-affinity Ca^{2+} binding sites. These values differ significantly from the stoichiometries quoted for DCCD incorporation [3,12]: whether this is a consequence of the different reactivities of the two carbodiimides, or a reflection of the different reaction conditions, remains a matter for further study. Further investigations (to be published elsewhere) show that the specifically-labelled sites of the ATPase show marked fluorescence changes in response to the binding of ligands. We have established that NCD-4 has considerable potential as a probe for carbodiimide-binding sites in proteins.

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