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# Membrane-specific inhibitors of the bovine heart mitochondrial ATPase

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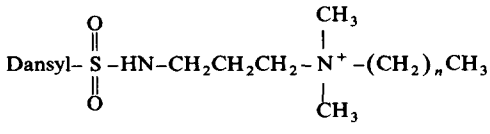
**New cationic inhibitors of the bovine heart mitochondrial ATPase have been synthesized by quaternizing 1-dansylamido-3-dimethypropylamine with decyl and hexadecyl iodides. These ligands are unique in their mode of action because they inhibit the submitochondrial membrane-associated forms of the enzyme more potently than the soluble form of the enzyme ($F_1$). Derivatives prepared with propyl or hexyl iodides are weak inhibitors and exhibit little affinity for submitochondrial membranes particle. The inhibitory effectiveness of these derivatives measured either in the direction of ATP synthesis or ATP hydrolysis results from efficient insertion into the membrane. Other inhibitory organic cations such as the 3:1 4,7-diphenyl-1,10-phenanthroline-ferrous chelate and alkyl guanidines inhibit both the membrane-associated and soluble ATPase comparably.**

## Introduction

The mitochondrial ATPase is inhibited by the cationic ligands octyl guanidine [1] and the 3:1 4,7-diphenyl-1,10-phenanthroline-ferrous complex [2–4]. These inhibitors block the submitochondrial-associated enzyme and the soluble enzyme at comparable concentrations. The coordination complex, and most likely the alkyl guanidines, bind to the $\beta$ subunit which composes part or all of the catalytic site [5,6].

In this communication, we wish to report the inhibitory properties of the danyslated organic cations of the general structure indicated below:



| *n* | Abbreviation |
|---|---|
| 0 | DD-0 |
| 2 | DD-3 |
| 5 | DD-6 |
| 9 | DD-10 |
| 15 | DD-16 |

DD-10 and DD-16 inhibit the ATPase associated with the submitochondrial particles about 50-times more potentially than the soluble ATPase ($F_1$). The $I_{50}$ values of these ligands for the membrane-associated ATPase activity are 7.9 and 3.4 $\mu$M, respectively; oxidative phosphorylation by submitochondrial particles is also inhibited by these ligands at similar concentrations. DD-3 and DD-6 are poor inhibitors of the membrane-associated ATPase with $I_{50}$ values of 1.4 and 0.4 mM, respectively. The efficiency of the ligands as inhibitors correlates with their ability to insert in the lipid bilayer of submitochondrial particles which has

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ETPH, submitochondrial particles.

been measured independently using either a fluorimetric or centrifugal assay.

## Materials and Methods

*Synthesis of inhibitors*

DD compounds were prepared by reacting iodoalkanes with dansyl-1′-dimethylaminopropylamine which was synthesized as follows. Dimethylaminopropylamine (1.8 g) and dansyl chloride were refluxed in 100 ml of acetone for 5 h. The acetone was removed on a rotary evaporator, and the residue was dissolved in chloroform and extracted twice with 2 vol. of 5% sodium carbonate, and once with 5% sodium bicarbonate. After washing the chloroform once with water, the organic phase was evaporated to dryness. The residue was recrystallized from methanol/water and dried in vacuo at 80°C. The light yellow crystals melted from 119–121°C.

DD-10 was prepared by stirring 1.0 g dansyl-1′-dimethylaminopropylamine with 2.4 g of iododecane in 100 ml of *N*,*N*-dimethylformamide for 40 h. Diethylether was added (approx. 1.01 g) until the cloud point, and the reaction mixture was placed in the freezer. The precipitate was harvested and recrystallized in acetone/diethylether. Analysis, calculated: %C 53.72; %H 7.68; %N 6.96; Fd: %C 52.54; %H 7.7; %N 7.00.

The other derivatives were prepared similarly; however, due to the difficulty in crystallizing the compounds, DD-3 and DD-6 were stored as an oil. All compounds exhibited a single spot when chromatographed on thin-layer silica gel plates, developed in isopropanol/ammonium hydroxide/water (9 : 1 : 2).

Solutions of the dansyl derivatives were prepared fresh by adding the iodide salt to a mixture of water and Dowex AG 1-X2Cl-resin forming the very soluble, but extremely hygroscopic chloride salt. The concentration of ligand was determined spectrophotometrically using an extinction coefficient of $4.8 \cdot 10^3\ M^{-1} \cdot cm^{-1}$.

*Enzyme preparations*

$F_1$ was isolated from bovine heart mitochondria and stored as an ammonium sulfate precipitate as described by Spitsberg and Blair [7]. The heavy mitochondria were obtained by a modification of the method of Smith [8] and were used to prepare submitochondrial particles (ETPH $Mg^{2+}$, $Mn^{2+}$) as described by Beyer [9]. For the assay of ATP synthesis the particles were used on the same day they were prepared, while for other assays the particles could be stored in liquid nitrogen for at least 1 week with little loss of activity. Submitochondrial particles depleted of $F_1$ were prepared by the method of Racker and Horstman [10].

*Enzyme assays*

All assays were carried out at 30°C. The ATPase activity of $F_1$ or ETPH particles was assayed by monitoring the formation of $P_i$ (Figs. 1 and 4) or by coupling the formation of ADP to the oxidation of NADH (Fig. 2) and measuring the decrease in the $A_{340}$ [11]. For the coupled assay, the reaction conditions were: 25 mM Tris-acetate (pH 7.6)/30 mM potassium acetate/10 mM magnesium acetate/2 mM phospho*enol*pyruvate/15 μg lactate dehydrogenase/100 μg pyruvate kinase/0.025 mg per ml ETPH particles/$1 \cdot 10^{-7}$ M FCCP. The uncoupler FCCP prevents the buildup of protonmotive force which could limit the rate of ATP hydrolysis. The reaction rate was determined after subtraction of the slow background rate in the absence of ATP. Controls were performed to insure that the coupling reactions were not rate-limiting under the conditions used. When larger quantities of enzyme were assayed for ATPase activity, the rate was determined by measuring the amount of $P_i$ liberated as a function of time using the method of Ohnishi and Gall [12]. An ATP-regenerating system was included, and for ETPH particles, FCCP was also present. In addition to the $F_1$ or ETPH particles, the final reaction mixtures contained: 25 mM Tris-acetate (pH 7.6)/30 mM potassium acetate/10 mM magnesium acetate/25 μg pyruvate kinase/4 mM phospho*enol*pyruvate/$1 \cdot 10^{-7}$ M FCCP (with ETPH particles)/5.0 mM ATP.

The succinate-driven synthesis of ATP by ETPH particles was measured by coupling the formation of ATP to the reduction of $NADP^+$, and quantitating the rise in the $A_{340}$ as a function of time. The final reaction conditions were: 25 mM Tris-HCl (pH 7.6)/25 mM glucose/50 mM sucrose/10 mM succinate/10 mM AMP/10 mM $KH_2PO_4$/

1.0 mM ADP (unless otherwise specified)/15 μg per ml glucose-6-phosphate dehydrogenase/215 μg per ml hexokinase/0.025 mg per ml ETPH particles. AMP in the reaction mixture prevented significant interference from adenylate kinase contamination. When necessary, the slow rate measured in the presence of oligomycin was subtracted.

*Other methods*

Fluorescence measurements were carred out with an Aminco-Bowman spectrofluorimeter equipped with a ratio photometer. Unless specified, the cell compartment was maintained at 30°C and semimicro cells having a working volume of 0.3 ml were used. The instrument was calibrated related to a quinine sulfate standard. 0.1 μg/ml in 0.1 M $H_2SO_4$ was given a relative fluorescence intensity of 180 of the units reported in the figures. ETPH particle concentrations are expressed as mg total protein per ml. Protein measurements were performed by the methods of Lowry et al. [13], using bovine serum albumin as a standard.

## Results

*Inhibition of membrane-associated ATPase*

The effectiveness of DD-3, DD-6, DD-10 and DD-16 as inhibitors of the ATPase associated with submitochondrial particles paralleled the length of the aliphatic chain (Fig. 1). At a fixed ATP concentration of 1 mM, the $I_{50}$ decreased from $1.4 \cdot 10^{-3}$ M for DD-3 to $3.4 \cdot 10^{-6}$ M for DD-16. The values for DD-6 and DD-10 were $4 \cdot 10^{-4}$ M and $7.9 \cdot 10^{-6}$ M, respectively. Under the conditions used, a Hill coefficient of 1 adequately described the kinetic data. When inhibition by DD-10 was examined over a range of ATP concentrations, linear double-reciprocal plots characteristic of a non-competitive pattern or a mixed one were observed; the $K_i$ determined from the slope of the plots was 4.4 μM if the concentration of the ligand in aqueous solution used in the calculation was corrected for ligand binding to the membrane (see below).

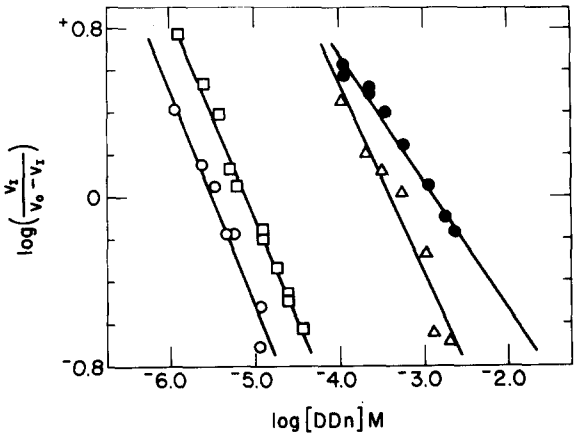



Fig. 1. Hill plots for the inhibition of the submitochondrial particles ATPase by DD-*n*. The ATPase activity was assayed as described under Materials and Methods. DD-16, ○; DD-10, □; DD-6, △; DD-3, ●.

*Assay of membrane insertion*

The dependence of the $I_{50}$ on the hydrophobicity of the inhibitor suggests a mechanism dependent on the insertion of the ligand into the membrane. Detailed studies with DD-10 have provided strong evidence that this is the case. Two methods were used to examine the insertion of DD-10 and the other dansyl derivatives into the lipid bilayer of submitochondrial particles. In one, the fluorescence increase attendant with the incorporation of the dansyl chromophore into a hydrophobic milieu was used to monitor its sequestration into the lipid bilayer. In the second, the depletion of ligand from the aqueous phase was measured after submitochondrial particles were removed from the solution by centrifugation. The concentration of ligand in the supernatant was measured spectrophotometrically using the 340 absorption maximum ($4.8 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$) of the dansyl chromophore.

Both these experimental methods can be used to demonstrate that membrane insertion exhibits saturable binding. The centrifugal method is most reliable in quantitating the equilibrium because it is a simple mass measurement. No assumptions regarding quantum yields or number of binding sites in the submitochondrial membranes are required, nor is it essential to have one reactant in excess relative to another for equilibrium constants to be calculated. To gather binding data, the ligand is incubated with submitochondrial particles which are then pelleted in an ultracentrifuge. The concentration of DD-10 remaining in the supernatant

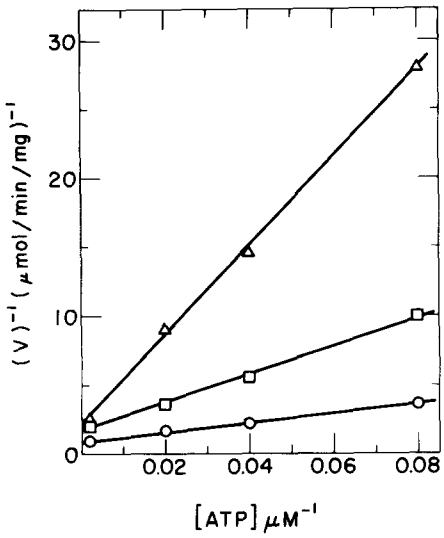



Fig. 2. Double-reciprocal plot of the inhibition of the submitochondrial particle ATPase by DD-10. The ATPase assays were performed as described under Materials and Methods. Control, ○; DD-10, 8.1 $\mu$M, □; DD-10, 36 $\mu$M, △.

can then be measured spectrophotometrically or fluorimetrically and the concentration of ligand sequestered in the particles calculated by subtracting this value from the total concentration of DD-10 added. The data at different ligand and submitochondrial particle concentrations (Fig. 2) are readily accounted for by a Scatchard analysis (Eqn. 1) where $K_d$ is the dissociation constant (in $\mu$M), $L_a$ and $SL$ are the concentrations of free and bound ligand, respectively (in $\mu$M), $S_t$ is the total concentration of submitochondrial particles (in mg/ml), $n$ is the number of nanomoles of ligand inserted per milligram of submitochondrial particles (nmol/mg) and $V$ ($V = SL/S_t$) is:

$$V = n - VK_d/L_a \quad (1)$$

The value of the dissociation constant (7.3 $\mu$M), derived by plotting the data according to Eqn. 1 (Fig. 3), agrees with that measured using fluorescence with ligand in excess to submitochondrial particles (data not shown).

The value of $n$ obtained from the Scatchard analysis is 178 nmol ligand per mg of protein. Since submitochondrial particles are 70% protein and 30% lipid, $n$ can be alternatively expressed as 415 nmol ligand per mg of submitochondrial particle lipid [14] which corresponds to 0.3 nmol ligand per nmol of lipid assuming a mean molecular weight of 800 for the phospholipid. Since the molar ratio of bound ligand to $F_1$ approaches 1000, neither the ATPase nor any other protein is responsible for the saturable binding of ligand in the membrane. As expected, DD-10 binds to both submitochondrial particles and $F_1$-depleted particles with identical affinities and exhibits indistinguishable fluorimetric properties. The quantum yield of DD-10 in membranes is 10-fold greater than that of the ligand in aqueous solution, but comparable to that for a dansyl moiety in a hydrophobic solvent or bound to bovine serum albumin.

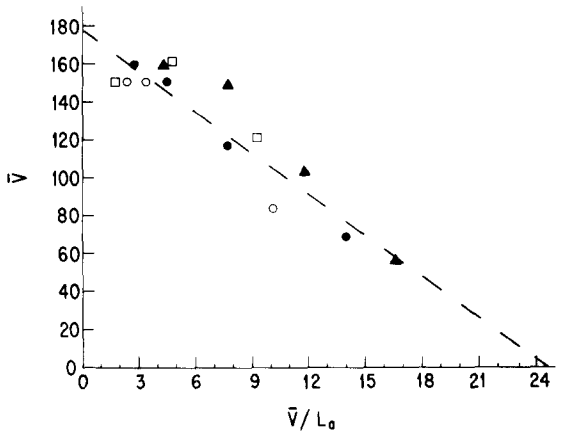



Fig. 3. Scatchard analysis of DD-10 binding to submitochondrial particles. The amount of DD-10 not bound to the particles was determined from that remaining in the supernatant after the particles were pelleted in the ultracentrifuge. The bound ligand was derived by subtracting the free ligand from the total added. Varying concentrations of submitochondrial particles were used: 0.1 mg/ml, □; 0.2 ml, ○; 0.3 mg/ml, ●; 0.4 mg/ml, ▲.

Centrifugation assays of the binding of the other dansyl derivatives were carried out in the expectation that the affinities for the membrane, like the inhibitory potency, would increase with the length of the hydrophobic chain. As expected, DD-16 was sequestered in the membrane far more effectively than DD-10. For example, in the presence of 0.16 mg/ml of submitochondrial particles, 98% of the DD-16 (56 $\mu$M) was inserted into the membrane. Under comparable conditions, 44% of the DD-10 was bound. However, at a total concentration of DD-6 or 30 $\mu$M, only 10% of the ligand has

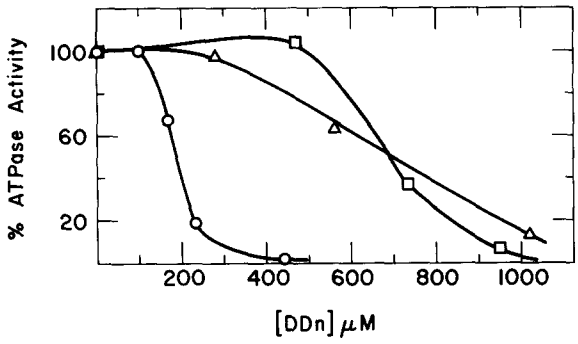



Fig. 4. Inhibition of the soluble $F_1$ ATPase by DD-*n*. The ATPase activity was assayed as described under Materials and Methods. DD-3, △; DD-6, □; DD-10, ○.

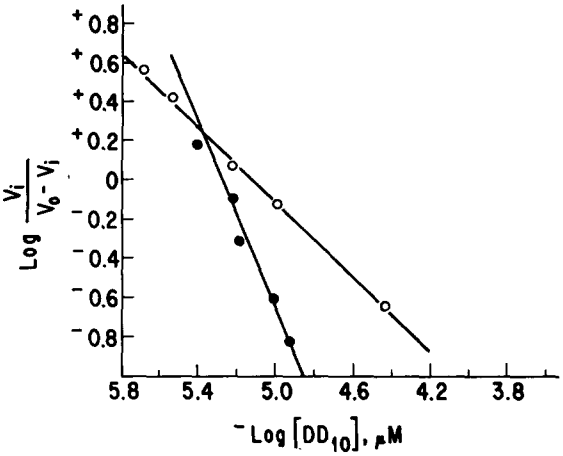



Fig. 5. Hill plots for the inhibition and hydrolysis in submitochondrial particles. The assays are described in Materials and Methods. ATP synthesis ●; ATP hydrolysis, ○.

inserted into the submitochondrial particles (0.16 mg/ml) compared to 65% for DD-10. The binding of DD-3 to the particles using the centrifugal assay was not observable in these concentrations ranges.

*Inhibition of $F_1$*

The soluble ATPase differs in its interaction with the DD-*n* ligands in two significant respects. First, it is less susceptible to inhibition by these ligands. For example, the $I_{50}$ of DD-10 for the soluble enzyme is 50-times higher than for the membrane-associated ATPase. Secondly, the concentration dependence of the inhibition is characterized by a Hill coefficient substantially greater than 1 (Fig. 4). These two features of the inhibition can be explained by our observation that DD-10 forms aggregates in the concentration range where inhibition takes place. Aggregate formation has been inferred from the abrupt derivation of the ligands fluorescence above 0.2 mM. This fluorescence quenching, which cannot be attributed to inner filter effects, is characteristic of the constraint of the chromophores in close association. Highly cooperative concentration dependencies are also features of micelle formation.

*Inhibition of oxidative phosphorylation*

Succinate-driven ATP synthesis by submitochondrial particles is also inhibited by DD-10. The inhibition is reversible and non-competitive with respect to ADP and $P_i$ (not shown). The Hill plot for the inhibition of the hydrolytic and synthetic activities is compared in Fig. 5. Two important features are apparent. The first is that the $I_{50}$ for both catalytic activities of the coupling factor are equivalent. On the other hand, the Hill coefficient for the inhibition of ATPase activity is 1, whereas that for the inhibition of synthase activity is 2. Several explanations are possible for this unanticipated observation. Protonmotive force could change the thermodynamics of inserting the ligand into the submitochondrial particles accounting for the change in the Hill coefficient. However, energization of submitochondrial particles with succinate before and after the addition of DD-10 failed to affect the extent of ligand binding as measured by fluorescence changes. The most likely explanation for the higher order dependence of ATP synthesis, may be that it reflects the inhibition of one or more of the oxidative enzymes responsible for generating protonmotive force from the oxidation of succinate. Independent assay of these enzymes has supported this suggestion.

## Discussion

The quaternary dansyl ligands reported here are the third example of reversible membrane active agents which affect the mitochondrial ATPase. The others are the general anesthetics, chloroform and halothane [15] and the lipophilic cation tetraphenyl arsonium [16]. The anesthetics act by uncoupling oxidative phosphorylation without col-

lapsing the proton gradient and activate the ATPase activity. In view of their enhancement of the catalytic activity, they probably do not bind to the active site but instead may interfere with essential conformational interactions between the $F_0$ and $F_1$ portions of the synthase. Precedent for the ability of anesthetics to perturb the conformation of membrane associated proteins is their preferential stabilization of the high-affinity conformer of the acetyl choline receptor protein [17–19]. Tetraphenyl arsonium, on the other hand, inhibits ATP synthesis only in mitochondria and not in submitochondrial particles and is postulated to act by binding to negative charges on the C-side of the mitochondrial membrane.

Although the precise mode(s) of interaction of DD-10 with the coupling factor cannot be fully specified with the data presently available, the $\beta$ subunit most likely contains the primary binding site for DD-10. This conclusion is based on three premises. First, the $\beta$ subunit contains the active site. Second, $F_1$, which is inhibited by DD-10, does not contain $F_0$ subunit. Finally, the $\beta$ subunit binds another hydrophobic cationic inhibitor, the 3 : 1 4,7-diphenyl-1,10-phenanthroline-ferrous ion complex.

In addition, if the binding site for the ligand is on the $\beta$ subunit, then the greater sensitivity of the membrane-associated enzyme can be rationalized. Using a novel bilirubin-dependent photooxidation, Hackney has demonstrated that the polypeptide chain of the $\beta$-subunit makes extensive contact with the membrane surface [20]. Since DD-10 efficiently inserts into the membrane, its cationic moiety must be at the membrane surface and contiguous to the $\beta$ subunit. Binding of the cationic group at a nonspecific site on the $\beta$ subunit would be greatly favored because of the high local concentration of the ligand in the submitochondrial particles.

Direct contact of DD-10 with the $F_0$ cannot be excluded. Inhibition of respiration in $F_1$ will not resolve this question because the ligand can inhibit the oxidative enzymes even in uncoupled mitochondria. The possibility that DD-10 inhibits the ATPase by perturbing membrane surface potential or the distribution of counterions is not consistent with its ability to inhibit the uncoupled ATPase activity and the ATP synthesis reaction at comparable concentrations.

## Acknowledgments

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