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INACTIVATION AND THE SITE OF LABELING OF F_1-ATPase FROM *MYCOBACTERIUM PHLEI* BY DICYCLOHEXYLCARBODIIMIDE, 7-CHLORO-4-NITROBENZO-2-OXA-1,3-DIAZOLE AND QUINACRINE MUSTARD

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The F_1-ATPase from *Mycobacterium phlei* is inactivated by dicyclohexylcarbodiimide (DCCD), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and quinacrine mustard. The inactivation is both time- and concentration-dependent and in the case of DCCD being more pronounced at acidic pH. The minimum inactivation half-time ($t_{1/2}$) for DCCD, NBD-Cl and quinacrine mustard was observed to be 14, 6 and 7 min, respectively. Inactivation of F_1-ATPase resulted in the incorporation of [^{14}C]DCCD as well as [^{14}C]NBD-Cl into α and γ subunits. The incorporation of label into α and γ subunits, utilizing [^{14}C]NBD-Cl, was reversible by dithiothreitol. Complete inactivation, by linear extrapolation to zero activity, revealed that 4 mol [^{14}C]DCCD and 4 mol [^{14}C]NBD-Cl bind per mol F_1-ATPase. Kinetic and binding studies show that these probes bind to site(s) distinct from ATP-binding site in F_1-ATPase from *M. phlei*.

Introduction

The proton-translocating ATPase complex in membranes from *Mycobacterium phlei* is composed of two sectors: an integral membrane protein, composed of three or more subunits and designated F_0, and an easily detachable peripheral membrane protein, composed of five different polypeptide subunits, designated F_1 [1], similar to that found in membranes of mitochondria, chloroplast and other bacterial species [2–4]. In recent years, a number of reagents known to react with specific amino acid residues have been utilized in an attempt to delineate essential amino acid residues of different subunits of F_0-F_1 ATPase complex involved in H^+-translocation and ATP synthesis [5–7]. Dicyclohexylcarbodiimide (DCCD), a well-known inhibitor of the membrane-bound ATPases, inhibits H^+-translocation and ATPase activity by binding specifically to one of the F_0 subunits, a DCCD-reactive proteolipid [2,8–10]. It has also been observed that DCCD, at an acidic pH, binds and inactivates F_1-ATPase isolated from mitochondria [11], *Escherichia coli* [12], chloroplasts [13] and thermophilic bacterium PS3 [14]. Furthermore, it has been shown that DCCD-binding site in these types of F_1 is located on the β-subunit.

Similarly, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) has been shown to inhibit soluble F_1-ATPase from chloroplasts [15], *Escherichia coli* [16] and *Rhodospirillium rubrum* chromatophores [17]. The binding location of NBC-Cl in *R. rubrum* F_1-ATPase has been localized on the β-subunit.

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Abbreviations: DCCD, dicyclohexylcarbodiimide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; Mes, 4-morpholineethanesulphonic acid; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

Our earlier studies [7] utilizing 2',3'-dialdehyde derivatives of ATP and ADP as affinity labels revealed that ATP-binding site is located on α-subunit while ADP-binding site is located on both α and β subunits of ATPase from *M. Phlei.* In an effort to understand the molecular mechanism of energy transduction, it is essential to delineate the structure and correlate with function.

The results presented in this paper show that DCCD at pH 6.0, NBD-Cl and quinacrine mustard inactivate F_1-ATPase from *M. phlei* in a time- and concentration-dependent manner. Studies also show that both [^{14}C]DCCD- and [^{14}C]NBD-Cl-binding sites are located on α and γ subunits of F_1-ATPase. Since ATP-binding site of *M. phlei* F_1-ATPase has been shown to be located on the α-subunit and the present results show that inhibition of ATP hydrolysis by DCCD, NBD-Cl and quinacrine mustard is uncompetitive, these results indicate that the binding site of these probes is distinct from ATP-binding site.

Materials and Methods

Preparation of membrane vesicles and solubilization of F_1-ATPase. M. phlei ATCC 354 was grown and harvested as described by Brodie and Gray [18]. Membrane vesicles were prepared by sonication of the cells followed by centrifugation at $144\,000 \times g$ for 60 min as described by Brodie [19]. F_1-ATPase was solubilized from the membrane vesicles by suspending either in 0.25 M sucrose or deionized water in the absence of inorganic ions followed by centrifugation at $105\,000 \times g$ for 90 min [20]. The resulting supernatant thus obtained contains ATPase activity. F_1-ATPase was purified from supernatant by affinity chromatography as described earlier [20].

Assay of latent ATPase. The ATPase activity in solubilized F_1-ATPase was determined after unmasking the latent ATPase by trypsin treatment [20]. The solubilized F_1-ATPase (1 mg/ml) in a buffer comprising 50 mM Tris-acetate/0.15 M KCl/4 mM $MgCl_2$ (pH 8.0), was treated with bovine pancreas trypsin (50 μg per mg protein) for 10 min at 30°C. After 10 min the reaction was terminated by the addition of soybean trypsin inhibitor (100 μg per mg protein). ATPase activity of the sample was determined in the presence of 4 mM $MgCl_2$ and 5 mM ATP at 37°C, by estimating the released inorganic phosphorus following the method of Fiske and SubbaRow [21].

The inhibition of F_1-ATPase activity by DCCD, NBD-Cl and quinacrine mustard was determined in unmasked (trypsin-treated) ATPase. Unless otherwise indicated, incubation with DCCD was carried out at pH 6.0.

Binding of [^{14}C]DCCD and [^{14}C]NBD-chloride to F_1-ATPase. F_1-ATPase was incubated with various concentrations of [^{14}C]DCCD (specific activity 54.6 Ci/mol) or [^{14}C]NBD-chloride (specific activity 100 C_i/mol) for indicated time-periods at 37°C in a total volume of 0.5 ml. The reaction was terminated with an equal volume of cold 10% trichloroacetic acid, followed by centrifugation in a Beckman-microfuge for 2 min at $10\,000 \times g$. The pellet thus obtained was washed twice with cold acetone. The pellet was solubilized in 20% SDS (v/v) containing 0.1 M NaOH and mixed with 5 ml of Aquasol (West Coast Scientific, La Jolla, CA) and the radioactivity was counted after neutralizing the pH with HCl.

SDS gel electrophoresis. Latent F_1-ATPase (100–200 μg protein) was incubated with either [^{14}C]DCCD (220 μM; Spec. Act., 1 nm = 2100 cpm) in 50 mM Mes-sodium hydroxide buffer (pH 6.0) or [^{14}C]NBD-chloride (200 μM, spec. act. 1 nmol = 2500 cpm) in 50 mM Tris-acetate buffer (pH 8.0) comprising 0.15 M KCl/4 mM $MgCl_2$ for a period of 2 h at 37°C. After the incubation, the reaction was terminated by the addition of 0.5 ml cold 10% trichloroacetic acid. The precipitate thus obtained was washed twice with cold acetone, dissolved in 80 μl of 0.1 M NaOH and subjected to polyacrylamide-SDS gel electrophoresis in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate as described by Kalra et al. [22]. The Coomassie blue stained gels were scanned at 555 nm using a Zeineh Soft Laser scanning densitometer. To determine the distribution of radioactivity in the gels, the gels previously fixed, stained and destained were sliced in 1 mm slices with a Bio-Red electric gel slicer. Slices were digested by overnight incubation in 400 μl of 30% H_2O_2 at 55–60°C and counted in 10 ml of aquamix scintillation fluid in a Beckman LS-8000 Scintillation counter.

Protein determination. The protein concentra-

tion of ATPase preparation was determined according to Lowry et al. [23].

Materials. [^{14}C]DCCD (54.6 Ci/mol) and [^{14}C]NBD-chloride (100 Ci/mmol) were purchased from Research Products International, IL; EEDQ, EDAC, quinacrine mustard, ATP (vanadate free), ADP, CTP from Sigma Chemical, St. Louis, MO. Low-molecular-weight protein standards, product No. 17-0446-01 (M_r range 14400–94000) were purchased from Pharmacia Fine Chemicals; Polyacrylamide, TEMED, ammonium persulfate and sodium dodecyl sulfate were from Bio-Rad. All other reagents used were of reagent-grade purity.

Results

Inactivation of F_1-ATPase by DCCD

Incubation of soluble F_1-ATPase from *M. phlei* with DCCD results in inactivation of ATPase activity as the pH decreased from 8.0 to 6.0 (Fig. 1); half maximal inhibition was around 7.0, while there was no inhibition above pH 8.0. The increased rate of inactivation at acidic pH probably reflects interaction of protonated carbodiimide with glutamate carboxylate anion of F_1-ATPase to form *O*-acylisourea [24]. Similar inactivation with DCCD has been observed for F_1-ATPase from *E. coli* and beef-heart mitochondria [11,12]. The in-

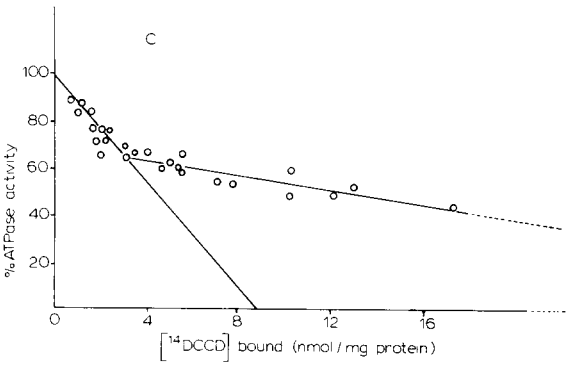


Fig. 2. Relationship between the binding of [^{14}C]DCCD and inactivation of F_1-ATPase activity. The data of [^{14}C]DCCD binding and the corresponding inhibition of ATPase activity were replotted to determine the relationship between the binding of [^{14}C]DCCD and inhibition of ATPase activity. Linear regression analysis was performed on a programmable Hewlett-Packard Comptuer 81 attached to a plotter to extrapolate all the points within the range 0–9 nmol [^{14}C]DCCD bound/mg protein to zero activity.

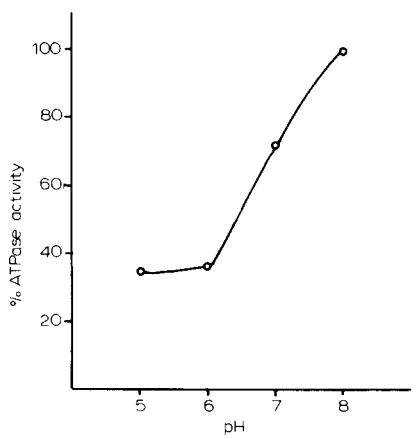


Fig. 1. The effect of pH on inactivation of F_1-ATPase by DCCD. Solubilized trypsinized F_1-ATPase (1 mg/ml) in a total volume of 500 μl was incubated at 37°C with DCCD (220 μM) at the pH values indicated for a period of 30 min. After the incubation period an aliquot of 200 μl was taken for the assay of ATPase activity as described under Materials and Methods. The buffers used were 50 mM Mes-NaOH (pH 5.0 and 6.0); and 50 mM Tris-acetate (pH 7.0 and 8.0). The ATPase activity at pH 8.0 was taken as 100%.

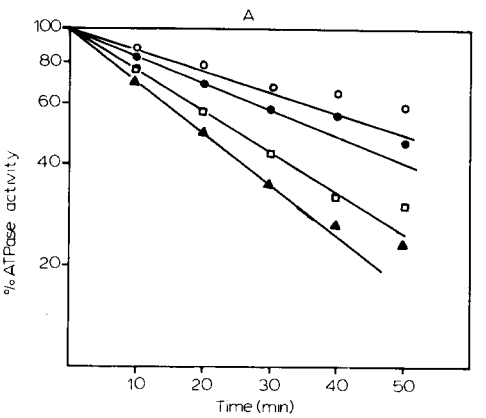


Fig. 3. Semilogarithmic plot of the inhibition of F_1-ATPase activity by various concentrations of DCCD. The data obtained from the time-course of the inhibition of F_1-ATPase activity of *M. phlei* was plotted on a semilogarithmic scale. Trypsinized F_1-ATPase (1 mg/ml) was incubated with various concentrations of DCCD in 50 mM Mes-NaOH buffer (pH 6.0) for the indicated time intervals at 37°C. After the incubation an aliquot of 200 μl was taken for assay of the ATPase activity in 500 μl 50 mM Tris-acetate buffer (pH 8.0) as described in Materials and Methods. [DCCD] (μM): 50 (○——○); 100 (●——●); 200 (□——□); and 400 (▲——▲).

activation and binding of [^{14}C]DCCD to F_1-ATPase from *M. phlei* was both time- and concentration-dependent (data not shown). As shown in Fig. 3, the inactivation of ATPase was pseudo-first-order for the initial 35–65% inactivation over a concentratoin range of 50–480 μM DCCD. This indicates that a kinetic measurement made within the first 60% of inactivation could be taken to represent an initial rate. Inactivation half-time ($t_{1/2}$) was calculated by determining the time required by DCCD to cause 50% loss of the F_1-ATPase activity. A plot of t versus $1/[DCCD]$ gave a straight line with the intercept of t, the minimum inactivation half-time of 14 min (data not shown). The plot of pseudo-first-order rate constants (K') vs. [^{14}C]DCCD bound shows that the reaction with respect to bound DCCD is of about first order (data not shown). Fig. 2 shows the relationship between binding of [^{14}C]DCCD and inhibition of ATPase activity. Extrapolation of the linear portion of the curve to 100% inactivation ATPase activity reveals that under these conditions 8.7 nmol [^{14}C]DCCD bind per mg F_1-ATPase corresponding to approx. 4 mol [^{14}C]DCCD bound per mol F_1. The value for the second portion of the curve was approx. 48 nmol [^{14}C]DCCD bound per mg F_1-ATPase. The latter value presumably represents low-affinity binding sites, which may not be specific.

Effect of glycine ethyl ester on the binding of [^{14}C]DCCD to F_1-ATPase

Since DCCD reacts with carboxyl groups and moderately with other residues in proteins, studies were undertaken to delineate whether the inactivation of F_1-ATPase by DCCD was due to modification of carboxyl group. This was ascertained by determining the binding of [^{14}C]DCCD and ATPase activity in the presence of glycine ethyl ester which is known to react selectively with carbodiimide-activated carboxyl group to yield stable carboxyl derivative [25]. As shown in Table I, the inactivation of F_1-ATPase by DCCD was not affected by the addition of glycine ethyl ester (0.2 M). However, binding of [^{14}C]DCCD was reduced to 50% in the presence of glycine ethyl ester, indicating that at least 50% of the residues with which DCCD reacts are carboxylic acid groups.

Effect of nucleotides on the inactivation of F_1-ATPase by DCCD

Studies carried out with TF_1-ATPase from thermophilic bacterium [14] have shown that nucelotides stimulated several-fold the inactivation of ATPase activity by DCCD. However, it has been shown that ATP, ADP and divalent cations protect F_1-ATPase from *E. coli* [12] against inactivation by DCCD. ADP and Mg^{2+} did not significantly prevent or enhance the inactivation of F_1-ATPase from *M. phlei* by DCCD (data not shown). However, CTP partially (30%) afforded protection against inactivation by DCCD (data not shown).

Inactivation of F_1-ATPase by carboxyl group reagents

Studies were carried out to determine whether carboxyl-group reagents other than DCCD, were effective in the inactivation of F_1-ATPase activity. As shown in Table II, EDAC and EEDQ carboxyl

TABLE I

EFFECT OF GLYCINE ETHYL ESTER ON INACTIVATION AND [^{14}C]DCCD BINDING IN F_1-ATPase

F_1-ATPase in 50 mM Mes-buffer (pH 6.0) was preincubated with [^{14}C]DCCD (100 μM) in the presence of glycine ethyl ester (200 mM) at 37°C for the indicated time intervals. After incubation aliquots were taken either to determine the ATPase activity or binding of [^{14}C]DCCD to F_1-ATPase as described under Materials and Methods.

Incubation time with [^{14}C]DCCD (min)	Without glycine ethyl ester		With glycine ethyl ester	
	%ATPase activity	Bound [^{14}C]DCCD (mol/mol F_1)	% ATPase activity	Bound [^{14}C]DCCD (mol/mol F_1)
10	68	0.036	73	0.027
40	65	0.047	69.5	0.026

TABLE II

INACTIVATION OF F_1-ATPase BY CARBOXYL-GROUP REAGENTS

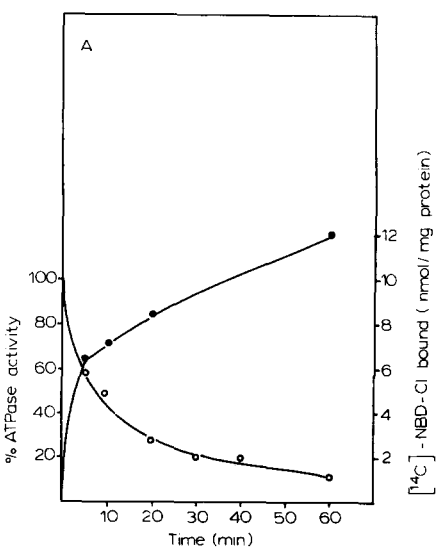
F_1-ATPase was preincubated at 37°C for 30 min with the carboxyl group reagents in 50 mM Mes-buffer (pH 6.0). After preincubation, aliquots of samples were diluted 5-fold in 50 mM Tricine buffer (pH 8.0) and immediately assayed for ATPase activity as described under Materials and Methods.

Reagents	Concentration (μM)	% ATPase activity inhibition
DCCD	100	44
	220	58
EDAC	200	36
	500	52
EEDQ	50	56
	100	67

group modifying agents inhibited F_1-ATPase activity to the same extent as DCCD.

Interaction of NBD-Cl with F_1-ATPase

The reaction of F_1-ATPase with NBD-Cl, at pH 7.4, resulted in a time-dependent inactivation of ATPase (Fig. 4A and B). The kinetics of inactivation showed that the rate of inactivation followed first-order kinetics (Fig. 4C) with a half-time of inactivation of 6 min (data not shown). As shown



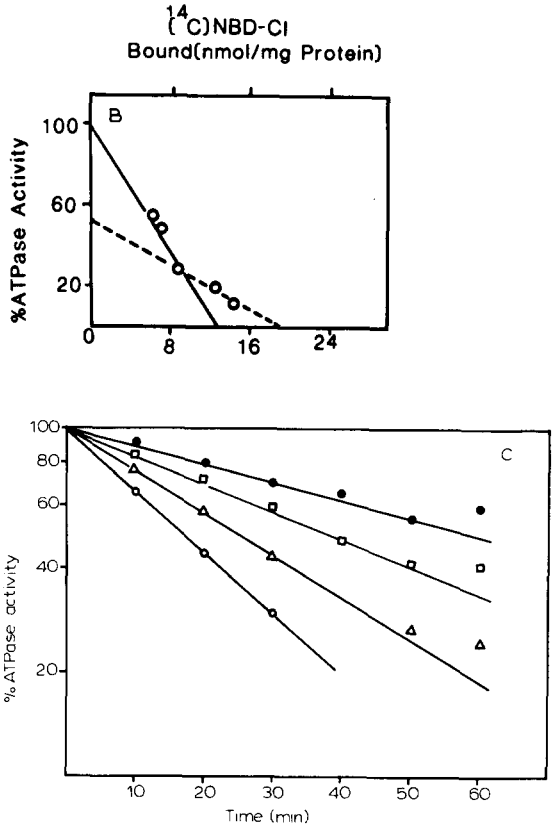


Fig. 4. (A) Time-course of the incorporation of [^{14}C]NBD-Cl in F_1-ATPase during its inactivation with [^{14}C]NBD-Cl. The reaction mixture in a total volume of 500 μl contained F_1-ATPase (1 mg/ml), a buffer comprising 50 mM Tris-acetate/0.15 M KCl/4 mM $MgCl_2$ (pH 8.0)/200 μM [^{14}C]NBD-Cl, was incubated at 37°C for the indicated time period. The reaction was terminated by 500 μl of 10% cold trichloroacetic acid for assay of binding of [^{14}C]NBD-Cl (●———●). The radioactivity associated with the washed precipitated protein was dissolved in 0.1 M NaOH and counted in a liquid scintillation counter, after neutralization. Trypsinized F_1-ATPase was treated with NBD-Cl (200 μM) in a total volume of 500 μl in the same buffer for indicated time intervals at 37°C, to determine the inactivation of F_1-ATPase with NBD-Cl (○———○). After various time periods, an aliquot of 200 μl was taken for the assay of ATPase activity as described in Materials and Methods. (B) The data of [^{14}C]NBD-Cl binding and the corresponding inhibition of F_1-ATPase activity were replotted to determine the relationship between the binding of [^{14}C]NBD-Cl and inhibition of F_1-ATPase activity. Linear-regression analysis was performed on a programmable Hewlett-Packard computer 81 as described in the legends to Fig. 2C. (C) Semilogarithmic plot of the inhibition of F_1-ATPase activity by various concentrations of NBD-Cl. The data in (A) (and other data not shown in (A)) were plotted on a semilogarithmic scale [NBD-Cl] (μM): 50 (●———●); 100 (□———□); 150 (△———△) and 200 (○———○).

in Fig. 4A, the rate of inactivation follows the rate of binding of [^{14}C]NBD-Cl with 80% inhibition obtained when 9.5 nmol NBD-Cl bound per mg protein or approx. 4 mol [^{14}C]NBD-Cl bound per mol of F_1-ATPase, utilizing a molecular weight of F_1-ATPase as 400000 [1]. It is also shown that the binding slowly continues even though the enzyme becomes fully inactivated. The inactivation as well as binding of [^{14}C]NBD-Cl to F_1-ATPase was observed to be reversed upon addition of dithiothreitol (data not shown).

Interaction of quinacrine mustard with F_1-ATPase

Recent studies of Laikind and Allison [26] have shown that quinacrine mustard (500 μM) causes potent inactivation of F_1-ATPase from beef-heart mitochondria. Their studies [27] revealed that local anesthetics, e.g., chloropromazine and tetracaine, protected F_1-ATPase against inactivation by di-

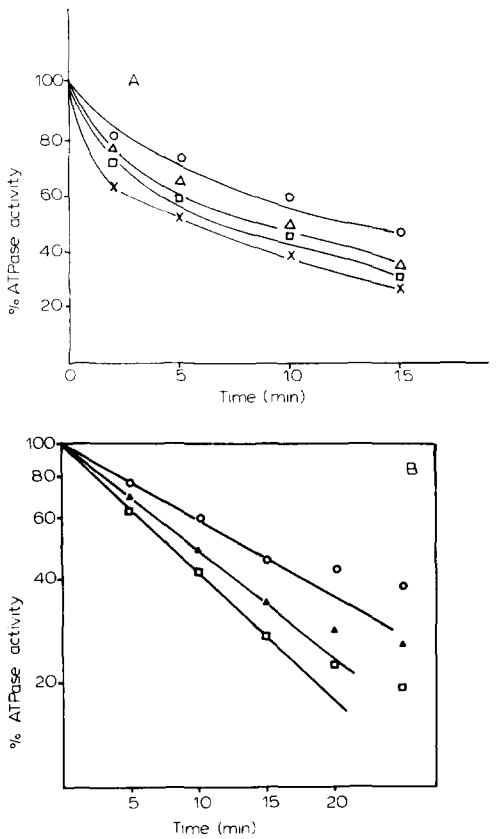


Fig. 5. (A) Time-course of inhibition of F_1-ATPase activity of *M. phlei* by quinacrine mustard. Trypsinized F_1-ATPase (1 mg/ml) was incubated with various concentrations of quinacrine mustard in a total volume of 500 μl comprising 50 mM Tris-acetate buffer (pH 8.0)/0.15 M KCl/4 mM $MgCl_2$ for the indicated time intervals at 37°C. After the incubation an aliquot of 200 μl was taken for the assay of the ATPase activity as described in Materials and Methods. [Quinacrine mustard] (μM): 20 (○———○); 50 (△———△); 100 (□———□) and 200 (x———x). (B) Semilogarithmic plot of the inhibition of F_1-ATPase activity by various concentrations of quinacrine mustard. The data in Fig. 5A were replotted on a semilogarithmic scale. [Quinacrine mustard] (μM): 20 (○———○); 50 (△———△) and 200 (x———x).

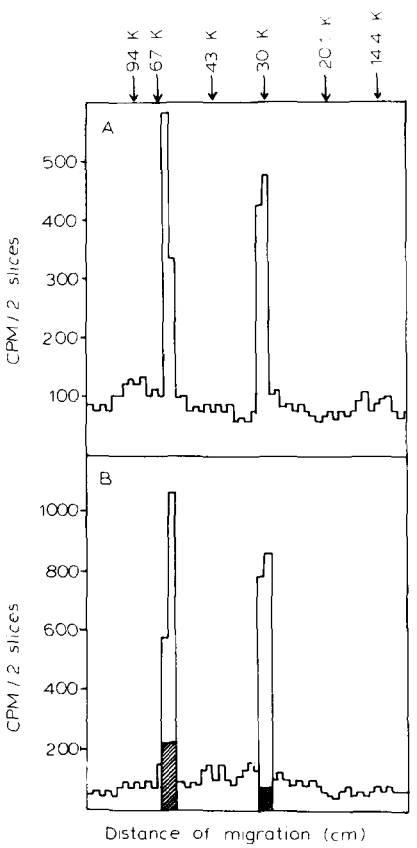


Fig. 6. Radioactive incorporation profile of F_1-ATPase labeled with either [^{14}C]DCCD (A) or [^{14}C]NBD-Cl (B): SDS-gel electrophoresis of [^{14}C]DCCD and [^{14}C]NBD-Cl labeled F_1-ATPase was carried out as described in Materials and Methods. Unshaded area represents the incorporation of radioactivity due to specific labeling of either [^{14}C]DCCD or [^{14}C]NBD-Cl, while the shaded area represents the labeling [^{14}C]NBD-Cl in the presence of 5 mM dithiothreitol. The arrows indicate the relative migration of the molecular weight standards (M_r range 14.4–94 K): α-lactalbumin, 14.4 K; soybean trypsin, 20.1 K; carbonic anhydrase, 30 K, ovalbumin, 43 K; bovine serum albumin, 67 K; and phosphorylase *B*, 94 K, obtained from Pharmacia Fine Chemicals, Sweden.

cyclohexylcarbodiimide; however, quinacrine, a structural analogue of chloropromazine, did not afford protection against inactivation by DCCD [27]. Since local anesthetics, tetracaine and lidocaine, did not prevent the inactivation of *M. phlei* F_1-ATPase by DCCD (Ref. 20 and Agarwal, N. and Kalra, V.K., unpublished data), it appears that molecular structure of F_1-ATPase from *M. phlei* is different from that of F_1-ATPase from *E. coli* and beef heart mitochondria. In an effort to obtain more insight into the molecular structure of F_1-ATPase from *M. phlei,* we studied the effect of quinacrine mustard, a covalent analogue of quinacrine. As shown in Fig. 5A, quinacrine mustard exhibits time- and concentration-dependent inactivation of F_1-ATPase from *M. phlei*. The pseudo-first-order rate constant, k_1, for the inactivation of F_1-ATPase by 200 μM quinacrine mustard is 0.086 min^{-1} at 37°C, (Fig. 5B) with half-time of inactivation ($t_{1/2}$) of 7 min (data not shown).

Identification of the [^{14}C]DCCD and [^{14}C]NBD-Cl binding subunit of F_1-ATPase

To ascertain to which subunit of F_1-ATPase [^{14}C]DCCD and [^{14}C]NBD-Cl bind covalently, the enzyme after inactivation by these agents was analyzed by SDS-gel electrophoresis and incorporation of radioactivity profile. The profile of radioactivity showed incorporation of [^{14}C]DCCD into two protein peaks corresponding to α subunit M_r 64000 and γ subunit M_r 33000 (Fig. 6A). Similarly, [^{14}C]NBD-Cl was found to covalently label α and γ subunit of F_1-ATPase from *M. phlei* which was substantially reduced in the presence of dithiothreitol, indicating that binding is specific (Fig. 6B). Comparison of the results of binding of [^{14}C]DCCD and [^{14}C]NBD-Cl to F_1-ATPase from several sources (representative data compiled from published results to *M. phlei* ATPase) shows firstly that in *M. phlei* ATPase there are 4 mol of either DCCD or NBD-Cl bound per mol of F_1-ATPase, in contrast to 1 or 2 mol reagent bound per mol F_1-ATPase from other sources (Table III). Secondly, the binding of either [^{14}C]DCCD or [^{14}C]NBD-Cl occurs in α and γ subunit of *M. phlei* ATPase, in contrast to binding to β subunit of F_1-ATPase from several other sources except *E. coli* and yeast mitochondrial ATPase, where the binding of [^{14}C]NBD-Cl has also been observed in α and β and α, β, γ and δ subunits, respectively. This difference in binding of [^{14}C]NBD-Cl and [^{14}C]DCCD to *M. phlei* F_1-ATPase in comparison to other ATPase may be due to switch in functions of subunits or different amino acid sequence in this F_1-ATPase compared to other ATPases. This is substantiated by the observation that ATP-binding site in *M. phlei* ATPase [7] has been observed to be in the α subunit, while in beef-heart mitochondrial ATPase this is in β-subunit [28]. Since ATP-binding site is located in α subunit of F_1-ATPase from *M. phlei,* it was of interest to determine whether DCCD or NBD-Cl modified ATP-binding site, thus affecting ATPase activity. Studies showed that DCCD, NBD-Cl and quinacrine mustard exhibit uncompetitive inhibition in the hydrolysis of ATP (data not shown).

TABLE III

COMPARISON OF STOICHIOMETRY OF BINDING AND SUBUNIT LABELING OF VARIOUS F_1-ATPases WITH [^{14}C]DCCD AND [^{14}C]NBD-Cl

Source of F_1-ATPase	Reagent DCCD		Reagent NBD-Cl	
	Subunit bound	mol per mol F_1	Subunit bound	mol per mol F_1
M. phlei	α and γ	4	α and γ	4
E. coli	β (Ref. 12)	1	β (Refs. 16 and 30) α and β (Ref. 31)	1
Beef-heart mitochondria	β (Ref. 11)	2	β (Ref. 32)	–
Thermophilic bacterium PS_3	β (Ref. 14)		–	–
R. rubrum chromatophores	β (Ref. 29)	1	β (Ref. 17)	1
Spinach chloroplasts	β (Ref. 13)	2	β (Ref. 15)	–
Yeast mitochondria	–	–	β (Ref. 33), also α, γ and δ	–

Discussion

The results presented in this study show that F_1-ATPase from *M. phlei* is inactivated by the carboxyl group reagents DCCD, EEDQ and EDAC. The inactivation is both time- and concentration-dependent and also pH-dependent, being more marked at acidic pH as has been observed with all other tested F_1-ATPases [11–13]. However, F_1 sector is relatively less sensitive to DCCD than the F_0 sector of the F_0-F_1 ATPase complex [8,9]. The correlation between the binding of [^{14}C]DCCD to F_1-ATPase and the inhibition of ATPase activity reveals that, up to 50% inhibition, the binding of DCCD is linear to the inhibition observed. The extrapolation of the linear portion of the curve to 100% inactivation of ATPase activity revealed that 4 mol of [^{14}C]DCCD bind per mol of F_1-ATPase. Moreover, glycine ethyl ester, a nucleophile which reacts selectively with carbodiimide-activated carboxyl groups yielding a stable carboxyl derivative, was found to prevent the incorporation by 50% of [^{14}C]DCCD in F_1-ATPase, but did not avert the inactivation of F_1-ATPase by DCCD. Thus 2 mol of [^{14}C]DCCD, which bind to reactive groups in F_1-ATPase, are carboxyl residue. However, it is not clear whether inactivation of ATPase is due to the binding of DCCD to these carboxylic acid groups. Similar results have been observed in the inactivation and binding of [^{14}C]DCCD to beef-heart mitochondrial F_1-ATPase [11]. These investigators [11] also observed 50% diminution in the binding of [^{14}C]DCCD, in the presence of glycine ethyl ester, while ATPase activity remained unaffected. Since the primary product that is formed when the carboxylate anion reacts with DCCD is an *O*-acylisourea [14,34], which can undergo number of rearrangements, when present in a protein in aqueous solution, to form a stable *N*-acylurea or react with neighboring lysyl side-chain to form a stable amide, the correlation of the inhibition of ATPase activity to [^{14}C]DCCD incorporated does not reflect the accurate stoichiometry of DCCD-ATPase interaction.

The covalent binding of [^{14}C]DCCD to F_1-ATPase followed by SDS-gel electrophoresis revealed that both α and γ subunit of ATPase are involved in the binding of DCCD. Since our previous studies [7] utilizing 2′,3′-dialdehyde derivative of ATP showed that ATP binding site was located on the α-subunit we determined whether DCCD and ATP shared the common site. Our results show that binding of [^{14}C]DCCD to α-subunit is not diminished in the presence of ATP, and also hydrolysis of ATP in the presence of DCCD is uncompetitive, indicating that [^{14}C]DCCD- and ATP-binding sites are not shared in F_1-ATPase from *M. phlei*. It is pertinent to mention that the DCCD-binding site is located on the β-subunit of F_1-ATPase from beef-heart mitochondria and *E. coli* [11,12].

The F_1-ATPase from *M. phlei* in common with the other tested F_1-ATPase [16,28–31] is also inactivated by NBD-Cl in a dithiothreitol-reversible manner. The inactivation correlates with binding of 4 mol NBD-Cl per mol F_1. SDS-gel electrophoresis of [^{14}C]NBD-Cl-labeled F_1-ATPase revealed that covalent binding occurred in both α and γ subunits, as has been observed with the covalent labeling with [^{14}C]DCCD. Approx. 80% of the [^{14}C]NBD-label in the α subunit and 90% in γ subunit was released when NBD-F_1-ATPase was incubated in the presence of dithiothreitol. These results suggest that the inactivation of F_1-ATPase was the result of the covalent modification of group in α and γ subunits to produce a labile NBD-Cl derivative. Spectroscopic studies [32] of interaction of NBD-Cl with various amino acids have revealed that sulfhydryl reagents cleave the NBD moiety from the labile tyrosyl O-NBD bond, whereas the more stable NH-NBD bonds are not affected. Thus our results indicate that NBD-Cl presumably modify tyrosine residues in the α and γ subunit of F_1-ATPase from *M. phlei*.

Moreover, the covalent binding of [^{14}C] NBD-Cl to F_1-ATPase was not reduced in the presence of ATP, suggesting that ATP and NBD-Cl do not share common binding sites. This is also substantiated by the observation that hydrolysis of ATP in the presence of NBD-Cl was observed to be uncompetitive. In F_1-ATPase from yeast mitochondria the covalent binding of NBD-Cl did not prevent binding of either Mg^{2+}-ATP or 8-azido-ATP, indicating that nucleotide-binding sites are not common with NBD-Cl-binding site [33].

Quinacrine mustard, an alkylating derivative of potent local anesthetic, quinacrine, was found to

inactivate F_1-ATPase from *M. phlei* in a time- and concentration-dependent manner. The half-time ($t_{1/2}$) of inactivation by 200 μM quinacrine mustard was 7 min. Recently, Laikland and Allison [26] also observed inactivation of MF_1-ATPase from beef heart mitochondria with quanacrine mustard. In these studies, they observed that ADP protected the enzyme against inactivation by quinacrine mustard. However, the presence of ADP did not afford protection against inactivation by quinacrine mustard in the case of F_1-ATPase from *M. phlei.* These results suggest that binding of quinacrine mustard to F_1-ATPase from *M. phlei* is different from that of beef-heart mitochondrial ATPase. However, further studies are needed to define the site(s) of binding of quinacrine mustard.

It is therefore concluded that there are at least two different binding sites for NBD-Cl and DCCD on the F_1-ATPase from *M. phlei,* one that is located on the α- and the other on the γ-subunit. It appears that the structure of F_1-ATPase from *M. phlei* is different from other F_1-ATPase wherein the binding of DCCD and NBD-Cl has been observed mostly on the β-subunit.

Since the stoichiometry of subunits are $\alpha_3\beta_3\gamma\delta\varepsilon$ in F_1-ATPase from *M. phlei* [1], it is possible that one molecule of probe binds to each subunit, i.e., three α and one γ subunit. Moreover, the binding site(s) of these probes is distinct from ATP-binding site, since the presence of ATP did not reduce the binding of either NBD-Cl or DCCD. These probes should be a valuable tool in delineating the primary structure of F_1-ATPase and to shed light on the molecular mechanism of energy transduction.

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