

Diamide blocks H^+ conductance in mitochondrial H^+ -ATPase by oxidizing F_B dithiol

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Effects of diamide on proton conductance of electron transport particles (ETPH), purified H^+ -ATPase (F_1 - F_0), F_0 of the H^+ -ATPase from beef heart mitochondria and binding of cadmium (^{109}Cd) to the H^+ -ATPase have been examined in the present paper. When ETPH and purified H^+ -ATPase are treated with 1 mM diamide, ATP-dependent generation of membrane potential, monitored by the absorbance change produced by the redistribution of oxonol VI, is consistently inhibited. Diamide also blocks passive H^+ conductance driven by a K^+ diffusion potential in the membrane sector, F_0 , of H^+ -ATPase. Furthermore, diamide treatment drastically reduces the binding of $^{109}\text{Cd}^{2+}$ to H^+ -ATPase, showing competition for the F_B dithiol group.

Diamide Cadmium Proton conductance Sulfhydryl group F_0 H^+ -ATPase Mitochondria

1. INTRODUCTION

Mitochondrial ATP synthase (H^+ -ATPase or F_0 - F_1) consists of an extrinsic water-soluble multi-protein complex (F_1) with ATPase activity and a membrane moiety (F_0) which exhibits proton conductance [1]. An understanding of the mechanism of proton conductance in the membrane sector is essential for elucidating the mechanisms underlying the utilization of the electrochemical proton gradient ($\Delta\mu H^+$) for ATP synthesis in mitochondrial membranes. One approach to the exploration of the mechanism uses protein-modifying reagents, prominent among which has been dicyclohexylcarbodiimide. This compound reacts with a carboxyl group in the mitochondrial proteolipid or the c subunit of *E. coli* F_0 [2]. Since several of the bovine F_0 subunits contain thiol groups which may or may not be functionally involved in proton translocation, another approach lies in the use of thiol-modifying reagents. One such subunit, containing a juxtaposed dithiol, is coupling factor B (F_B) [3]. F_B -dependent reactions involving isolated F_B [4-6], F_B in F_0 - F_1 [7,8] and F_B in F_0 [9] are in-

hibited by Cd^{2+} and phenylarsine oxide under conditions where the monothiol groups are protected by excess 2-mercaptoethanol. Monothiol reagents such as *p*-hydroxymercuribenzoate, iodoacetate and *N*-ethylmaleimide also inhibit F_B -dependent ATP-driven reactions [6]. On the other hand, long-chain maleimides block H^+ conductance at a site which would appear not to involve F_B [10]. Thiol-oxidizing reagents, e.g. diamide [11] and cupric *o*-phenanthroline [7], have similar inhibiting effects on isolated F_B and the ATP-dependent activities of purified H^+ -ATPase.

Here we extend the use of thiol-modifying reagents, particularly diamide, to the study of passive H^+ conduction in purified F_0 and compare the response to that in phosphorylating submitochondrial particles (ETPH) and H^+ -ATPase. The site of action of the thiol oxidant, diamide, has been determined.

2. MATERIALS AND METHODS

Electron transport particles (ETPH) were prepared from frozen beef heart mitochondria and ex-

tracted with lysolecithin to obtain a purified vesicular preparation of H^+ -ATPase (F_1 - F_0) [12]. The H^+ -ATPase was treated with 3.5 M NaBr for the preparation of the membrane sector F_0 [3]. ATP-driven membrane potential was monitored indirectly by measuring the absorbance change of the voltage-sensitive dye, oxonol VI, at 594 minus 630 nm as described by Pringle and Sanadi [8], using a Perkin-Elmer model 557 dual-wavelength spectrophotometer. F_0 was reconstituted into K^+ -loaded asolectin liposomes by a freeze-thaw-sonication method as described in [9] with minor modifications as follows: after thawing, the external potassium was removed by centrifugation at $50\,000 \times g$ for 30 min. in a Beckman model L5-50 ultracentrifuge, rotor Ti50. The pellet of F_0 -proteoliposomes was resuspended in 10 mM Tricine-NaOH, pH 7.5, containing 0.25 M sucrose. Passive proton conductance through F_0 -proteoliposomes was measured by the quenching of 9-aminoacridine fluorescence using a Perkin-Elmer MPF-44A spectrofluorometer with excitation and emission wavelengths of 365 and 451 nm, respectively. A potassium diffusion potential, which promotes K^+ efflux and a consequent flux of protons inward through the proton channel of the F_0 , was initiated by the addition of 50 ng valinomycin in 5 μ l ethanol. For radiolabeling of the H^+ -ATPase with ^{109}Cd , 1 mg enzyme (5 mg/ml) was incubated at 30°C for 30 min with or without 1 mM diamide, then 20 μ l of 2 mM CdCl_2 (6.25 $\mu\text{Ci}/\mu\text{mol}$) was added and the incubation continued at room temperature for an additional 10 min. The labeled H^+ -ATPase was loaded onto a Sephadex G-25 column (1 $\times$ 20 cm) pre-equilibrated with 5 mM Na_2HPO_4 , adjusted to pH 7.0. Fractions were collected and counted on a Beckman 5500 gamma counter. SDS-PAGE was carried out as described by Sanadi et al. [9]. Further details of the procedures are described in the figure legends.

Diamide [diazinedicarboxylic acid bis(*N,N*-dimethylamide)] and Sephadex G-25 (fine) were obtained from Sigma, St. Louis, MO, radioactive cadmium (^{109}Cd) from New England Nuclear, Boston, MA, and oxonol VI from Molecular Probes, Junction City, OR. Other chemicals were reagent grade.

3. RESULTS

3.1. Effect of diamide on membrane potential in ETPH and H^+ -ATPase vesicles

Membrane potential of the H^+ -ATPase vesicles was monitored by measuring the absorbance change caused by the redistribution of the voltage-sensitive dye oxonol VI. Fig.1 shows that the absorbance decrease brought about by the addition of ATP/Mg was inhibited significantly by treatment of the H^+ -ATPase with diamide. For comparison, data obtained with ETPH are also shown. 50% inhibition was obtained at around 0.3 mM diamide for H^+ -ATPase and around 0.7 mM for ETPH. Ammonia-EDTA extracted submitochondrial particles required about 1.5 mM diamide for similar inhibition [11]. It is interesting that the H^+ -ATPase preparation is more sensitive to inactivation by diamide than ETPH, since the opposite

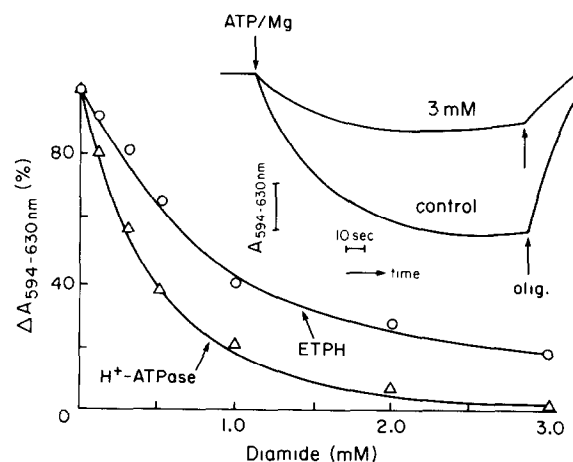


Fig.1. Effects of diamide on oxonol VI absorbance changes in energized ETPH and H^+ -ATPase. 500 μg ETPH or 100 μg H^+ -ATPase (1 mg/ml) was incubated with diamide as indicated in a solution containing 10 mM Tricine-NaOH, pH 7.5, and 0.25 M sucrose at 30°C for 30 min. After incubation, 100 μg ETPH or 50 μg H^+ -ATPase was added to 2 ml of assay medium containing 40 mM Tris-acetate, pH 7.5, 0.25 M sucrose, 1 mM MgCl_2 , and 1.5 μM oxonol VI. The proton gradient was generated by addition of 20 μ l ATP/Mg (1 : 1, 0.25 mM). Oligomycin (10 μ l of 100 $\mu\text{g}/\text{ml}$ ethanol) was added after the steady-state membrane potential was attained. The inset shows absorbance changes on adding ATP/Mg to the H^+ -ATPase with and without 3 mM diamide treatment. The arrow shows where oligomycin was added.

holds for inhibition by Cd^{2+} and phenylarsine oxide [5].

3.2. Effect of diamide on passive proton conductance of F_0

A more direct method for examining the effect of inhibitors on proton conductance is measurement of passive proton conductance through F_0 of the H^+ -ATPase reconstituted into K^+ -loaded liposomes (F_0 -proteoliposomes) (see section 2). The results obtained by this procedure are shown in fig.2A. It is clearly seen that the fluorescence quenching is markedly decreased by treatment of the F_0 with diamide (1–5 mM). The inhibition is evident both by the decrease in steady state of fluorescence quenching and the initial rate of quenching (proportional to the rate of H^+ conductance) (fig.2B). The quenching curves were analyzed in terms of a double-exponential decay by a reiterative non-linear regression procedure. From the experimental time constants, the initial quenching rate $Q_i = (\delta Q / \delta t)_{t=0}$ was calculated and plotted as a function of diamide concentration. The results indicated strongly that diamide blocked

proton conductance through the F_0 sector of the H^+ -ATPase. The blocking effect was not overcome by addition of oligomycin, DCCD or Cd^{2+} . These compounds also inhibited H^+ conductance independently (not shown). Thus, the results both on the inhibition of absorbance change of oxonol VI in F_0 - F_1 and the passive proton conductance in F_0 would further confirm that diamides does block, rather than enhance, the proton conductance of F_0 in the mitochondrial membranes.

3.3. Radiolabeling of H^+ -ATPase with ^{109}Cd

We showed previously that cadmium produces discharge of the ATP-driven membrane potential in the mitochondrial H^+ -ATPase and also blocks passive proton translocation through F_0 -proteoliposomes [9]. We have shown that treatment of the H^+ -ATPase or F_0 with the highly specific thiol-oxidizing agent, copper *o*-phenanthroline [13] decreased the binding of $^{109}\text{Cd}^{2+}$, under conditions where the protein-bound Cd^{2+} comigrated exclusively with coupling factor B in SDS-PAGE. Thus, it seemed significant to examine the effect of diamide on binding of cadmium to the H^+ -ATP-

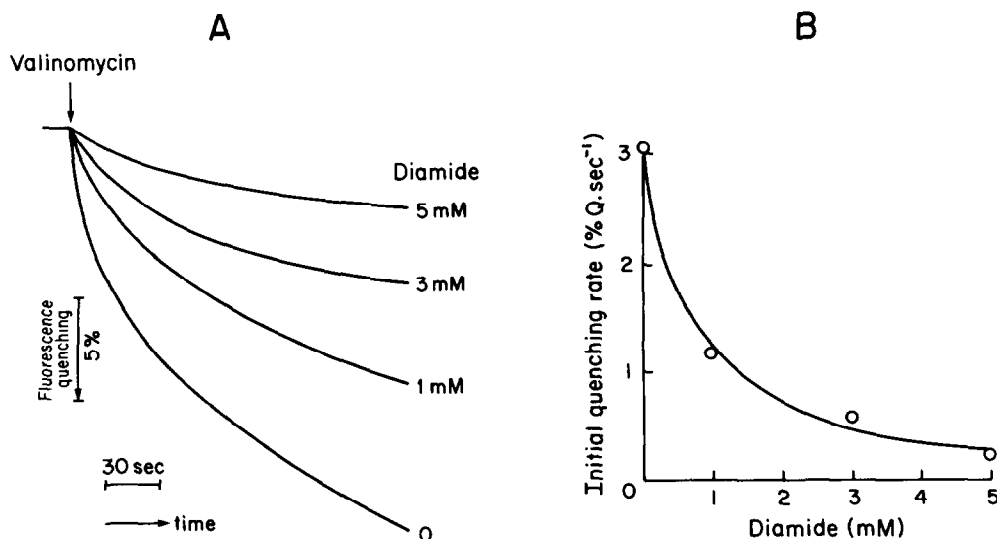


Fig.2. (A). Effect of diamide on passive proton conductance through F_0 . K^+ -loaded proteoliposomes, 20 μl (as described in section 2) containing 25 μg mitochondrial F_0 , were incubated at 30°C for 30 min in the absence (control) and presence of diamide. Then the samples were transferred to assay media containing 10 mM Tricine-NaOH, pH 7.5 0.25 M sucrose and 5 μM 9-aminoacridine. The traces show the quenching of the fluorescence after addition of 50 ng valinomycin. (B). The initial rates of H^+ conduction were calculated by a double-reciprocal decay procedure as in the text and plotted vs diamide concentration.

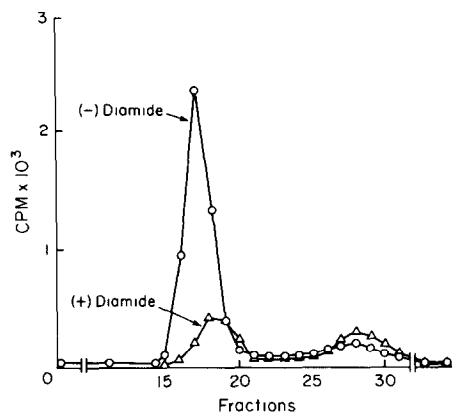


Fig.3. Binding of cadmium (^{109}Cd) to H^+ -ATPase in the presence and absence of diamide. The radiolabeling conditions are described in section 2. 1 mg (200 μl) of the H^+ -ATPase was loaded onto the Sephadex column. Each fraction (about 0.25 ml) was counted in a gamma counter.

ase. Separation of free Cd^{2+} from the protein-bound Cd^{2+} was performed by a Sephadex G-25 gel filtration procedure. Fig.3 shows the distribution of radiolabel bound to the H^+ -ATPase treated with diamide and the control without diamide. Prior treatment of the H^+ -ATPase with diamide reduced protein-bound $^{109}\text{Cd}^{2+}$ substantially.

Cd^{2+} also binds monothiols but weakly compared to binding to vicinal dithiols [6]. To decrease non-specific binding to monothiols, we have in the past routinely used a 5- or 10-fold excess of 2-mercaptoethanol in the reaction mixture [6]. In the experiments with diamide, however, use of mercaptoethanol in slight excess over diamide mostly prevented the inhibition caused by diamide (our data not shown, as well as data of Yagi and Hatefi [11]). These results show that unlike Cd^{2+} , diamide shows no preference for juxtaposed dithiols over monothiols, and it is not appropriate to consider the reagent specific to juxtaposed dithiols. In another experiment, 2-mercaptoethanol at 0.2 mM, which was less than the diamide concentration (1 mM) but more than the cadmium (40 μM), was added just before the addition of cadmium. The results were quite similar to those shown in fig.3.

The ^{109}Cd binding was measured in other experiments using 5 and 10 mM diamide with similar results. Treatment with lower diamide (0.1 mM),

however, had much less effect and reduced bound $^{109}\text{Cd}^{2+}$ by only 15%.

For further identification of the Cd-binding protein, Cd^{2+} -labeled H^+ -ATPase and F_B were separated on SDS-PAGE as in [9]. Both samples gave a single radioactivity peak with an M_r of 13 000 as in earlier studies [6,7,9] (not shown), which corresponds to the value obtained for the disulfide form of F_B [7]. By this method also, the labeling of the 13 kDa band was reduced when the H^+ -ATPase was treated with diamide before addition of Cd^{2+} (not shown).

4. DISCUSSION

Earlier studies had shown that diamide, a thiol-specific oxidant [14], inhibited the coupling factor activity of F_B [11], presumably by oxidizing the functionally essential dithiol group. The thiol oxidant also inhibited the ATP-dependent absorbance change of oxonol VI in submitochondrial particles [11] and purified H^+ -ATPase (fig.1).

In attempts to define the site of action of diamide more closely, effects on F_0 were studied. Passive H^+ conduction in F_0 is inhibited by diamide (fig.2) as shown by the quenching of 9-aminoacridine fluorescence. A recent preliminary report [15] also found inhibition of H^+ conduction in F_0 -proteoliposomes. While these authors also reported similar inhibition by diamide in AE-SMP, the opposite effect, viz. strong stimulation of H^+ conduction, was observed in E-SMP. The stimulated H^+ conduction was inhibited by oligomycin, DCCD and *N*-ethylmaleimide which restricted these effects to F_0 -mediated H^+ conductance. They attribute the inconsistent behavior of E-EMP to an $-\text{SH}$, different from F_B thiols, within the hydrophobic region of the membrane.

There is evidence for the presence of another $-\text{SH}$ in $\text{F}_0\text{-F}_1$, approx. 12 Å within the bilayer arising from a study of the effects of a series of *N*-polymethylenecarboxymaleimides (or acid maleimides or AM) in which the number of spacer methylene groups separating the polar and non-polar parts of the molecules varied from 1 to 11 [16]. The inhibition (and not stimulation) of energy-linked reactions of the H^+ -ATPase and F_0 H^+ conduction was maximal with $-(\text{CH}_2)_{10}$ or 11- and significantly greater than that obtained with *N*-ethylmaleimide. Since the binding of $^{115}\text{Cd}^{2+}$ to

H⁺-ATPase was not affected by prior treatment of the enzyme complex with AM10, F_B is not the protein modified by the inhibitor. The -SH modified by AM10 cannot be the -SH in E-SMP modified by diamide [15], since a stimulation of H⁺ conduction, unlike the strong inhibitory effect of AM10, is produced.

Thus, there is no satisfactory explanation for the stimulation in E-SMP by diamide. The inhibition of H⁺ conduction in AE-SMP, H⁺-ATPase and F₀ (fig.2) is most probably caused by modification of F_B dithiol. This is substantiated in F₀-F₁ by the competition between diamide and Ca²⁺ for the F_B modification shown in fig.3.

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