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N-Ethylmaleimide inhibition of the catalytic activities of the *Dunaliella salina* coupling factor 1 (CF_1) and the restoration of the inhibition of the CF_1 ATPase activity by *N*-ethylmaleimide

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The sensitivity of the catalytic activities of the *D. salina* chloroplast coupling factor 1 (CF_1) to chemical modification by *N*-ethylmaleimide has been investigated. (i) When *D. salina* thylakoid membranes are treated with *N*-ethylmaleimide, both photophosphorylation and the inducible CF_1 ATPase activity are partially (approx. 60%) inhibited. The inhibition of both activities does not require the presence of a proton-motive force, and the inhibition of photophosphorylation is directly related to the *N*-ethylmaleimide-covalent modification of CF_1 as shown by (a) the time-course for the inhibition and (b) the maximal extent of inhibition. (ii) Treatment of the purified, latent, *D. salina* CF_1 with low concentrations of *N*-ethylmaleimide also results in the partial (approx. 60%) inhibition of the inducible ATPase activity ($I_{50} \approx 50$ μM). The inhibition does not require the presence of the chemical modifier during the activation of the enzyme. (iii) *N*-ethylmaleimide-induced inhibition of the ATPase activity of either membrane-bound or solubilized CF_1 is partially reversed by either (a) prolonged incubation at low concentrations of *N*-ethylmaleimide or (b) short incubation times at high concentrations of *N*-ethylmaleimide. The results are interpreted as indicating multiple binding sites on the *D. salina* CF_1 that have different rates of reactivity with *N*-ethylmaleimide. Those sites (or site) that react rapidly with *N*-ethylmaleimide cause(s) an inhibition of both ATP synthase and ATPase activities, whereas those sites (or site) that react more slowly partially restore(s) the original ATPase activity. The effects of *N*-ethylmaleimide on the catalytic activity of *D. salina* CF_1 are probably mediated by *N*-ethylmaleimide-induced conformational changes of the enzyme.

Introduction

The energy-transducing H^+-ATP synthase complex (CF_0-CF_1) associated with chloroplast thylakoid membranes contains two functionally distinct parts, an intrinsic membrane portion (CF_0), whose function is to facilitate the movement of protons across the thylakoid membrane, and an extrinsic membrane portion (CF_1), whose function is to catalyze the synthesis of ATP using the energy in the protonmotive force [1]. The extrinsic portion of the complex, CF_1, can be readily released from thylakoid membranes by a variety of treatments, and contains, depending upon the method of extraction, from four to five different kinds of subunits, designated α, β, γ, δ and ϵ in order of apparent decreasing molecular weight when separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The most probable stoichiometry for the subunits is $\alpha_3\beta_3\gamma_1\delta_{1-3}\epsilon_1$ [2,3].

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Abbreviations: CF_1, chloroplast coupling factor 1; EDTA, ethylenediamine tetraacetic acid; Chl, chlorophyll.

Based on a number of observations, including the use of chemical modifiers [1] and the homology in the amino-acid sequence of subunits isolated from widely divergent species [4], it has been argued that the active site for the enzyme probably resides on the β-subunit, although some recent results might suggest that the active site is composed of portions of polypeptides from both the α- and β-subunits (at so-called 'interfaces') [5]. The function of the three smaller subunits remains equivocal, although it has been demonstrated in bacterial systems, in which an active ATPase can be reconstituted from nondenatured subunits, that the minimal complex for an active ATPase requires all three subunits, α, β, and γ [6].

McCarty and collaborators [7] have suggested that the γ-subunit may play a role in the gating of protons through CF_1. Central to their thesis is the observation that the chemical modification of various γ-subunit sulfhydryls with substituted maleimides can radically alter the proton permeability of thylakoid membranes [8]. The γ-subunit of the vascular plant CF_1 contains two sulfhydryls and one disulfide [9,10]. One of the sulfhydryls is apparently always accessible to modification by maleimides, the modification of which has no obvious effect on the coupling factor enzymatic activities [10]. The second sulfhydryl of the thylakoid-bound coupling factor γ-subunit is not readily accessible to chemical modification but can, however, be rendered more reactive upon the development of a transmittable protonmotive force [11,12]. When modified by monofunctional maleimides, proton flux across the membrane is inhibited [8]; however, when this sulfhydryl is cross-linked to the readily reactive sulfhydryl by bifunctional maleimides, proton flux is greatly accelerated [8]. Of interest to us has been how general this phenomenon is with coupling factors associated with other thylakoid membranes.

We have been studying the coupling factors of various species of the salt tolerant, unicellular, eukaryotic green algae *Dunaliella*. In many respects the *Dunaliella* coupling factors are very similar to their higher (vascular) plant counterparts. For example, as isolated, the ATPase activity of *Dunaliella* CF_1's are completely latent. A brief heat treatment induces a Ca^{2+}-dependent ATPase activity, whereas selected organic solvents and detergents induce a Mg^{2+}-dependent activity [13]. In addition, antisera directed against both the vascular plant spinach CF_1 and the eukaryotic, green algal *Chlamydomonas reinhardtii* CF_1 readily cross-react with the *Dunaliella* coupling factors [14]. Thus, the *Dunaliella* energy conservation apparatus appeared to be a good system in which to test the generality of the conformational sensitivity of coupling factors to maleimides.

In this contribution we show, however, that there are major differences between the sensitivity to maleimides for both the thylakoid-bound and soluble *D. salina* CF_1 when compared to the vascular plant CF_1. Photophosphorylation catalyzed by *D. salina* thylakoid membranes is rapidly inhibited by treatment of the membranes in the dark at low concentrations of *N*-ethylmaleimide. This inhibition is neither accelerated nor enhanced by the formation of a proton-motive force. The inhibition is not due to an indirect effect of *N*-ethylmaleimide on the *D. salina* electron-transfer chain, but rather we demonstrate that it is the direct result of inhibition by *N*-ethylmaleimide modification of the thylakoid-bound CF_1.

In a similar manner, the catalytic activity of the activated, soluble *D. salina* CF_1 is also inhibited by treating the latent coupling factor either in situ or in vitro with *N*-ethylmaleimide. The inhibition of the CF_1 ATPase activity by *N*-ethylmaleimide is only partial and is a function of both concentration and time. Low concentrations of *N*-ethylmaleimide ($I_{50} \approx 50\ \mu M$) or short incubation times ($t_{1/2} \approx 20$ s at 0–4°C) at higher concentrations result in a maximal inhibition of about 50–60%. This inhibition, however, is markedly reversed by either higher concentrations of *N*-ethylmaleimide or longer incubation times at lower concentrations, resulting in the restoration of approx. 80–90% of the original ATPase activity. These observations strongly suggest that there are multiple *N*-ethylmaleimide binding sites on CF_1 that markedly influence the catalytic activity of the enzyme.

Methods and Materials

Culture conditions. Cultures of *Dunaliella salina* were grown in the laboratory at room temperature in a medium described by Ben-Amotz et al. [15]. Culture flasks were stirred continually and il-

luminated with a combination of soft white fluorescent and incandescent lights. Small batch cultures (300 ml), used for the routine preparation of thylakoid membranes, were diluted 1 : 2 daily in order to maintain the cells in the log growth phase. Large batch cultures (5 L), used for the preparation of CF_1, were grown to stationary phase prior to harvesting the cells.

Preparation of Dunaliella salina thylakoid membranes. Cells from cultures in log phase were collected by centrifugation (500 × g; 5 min) and washed once in a medium containing 20 mM Tricine-NaOH (pH 8.0) and 15% (v/v) glycerol. The cells were osmotically shocked in 40 ml of buffer containing 20 mM Tricine-NaOH (pH 8.0)/10 mM NaCl/1.0 mM $MgCl_2$. The thylakoid membranes were pelleted by centrifugation (3000 × *g*; 5 min), thoroughly homogenized, and resuspended in a small volume (approx. 0.5 ml) of a buffer containing 20 mM Tricine-NaOH (pH 8.0)/10 mM NaCl/1.0 mM $MgCl_2$. The membranes were stored at 0–4°C until further use.

Treatment of thylakoid membranes with N-ethylmaleimide. Thylakoid membranes, equivalent to 300 μg Chl, were suspended in a total volume of 1.0 ml in buffer containing 20 mM Tricine-NaOH (pH 8.0)/10 mM NaCl/1.0 mM $MgCl_2$. *N*-ethylmaleimide was added and the suspension incubated in the dark (see figure and table legends for the exact incubation conditions). Reaction mixtures were quenched by the addition of two to four equivalents of dithiothreitol. The membranes were collected by centrifugation (12000 × *g*; 2 min; 4°C), washed once, and finally resuspended in 1.0 ml of the above buffer. The membranes were stored at 0 to 4°C until further use.

Photophosphorylation. Reaction mixtures for photophosphorylation contained, in a total volume of 1.0 ml, thylakoid membranes, equivalent to 10–20 μg Chl/20 mM Tricine-NaOH (pH 8.0)/5 mM $MgCl_2$/4 mM [^{32}P]phosphate (approx. $2 \cdot 10^6$ cpm)/3 mM ADP/50 μM phenazine methosulfate. The stirred suspension was illuminated for 30 s at 30°C with ultraviolet filtered, saturating white light, and esterified phosphate was determined as previously described [16].

ATPase activity of N-ethylmaleimide-treated thylakoid membranes. To 1.0 ml aliquots of *N*-ethylmaleimide-treated thylakoid membranes, 0.5 ml of cold (0°C) chloroform were added. The suspension was gently shaken for 15 s, the phases separated by centrifugation (12000 × *g*; 1.0 min), and the aqueous phase removed. The protein content of the aqueous phase was determined [17], and the ethanol-induced, Mg^{2+}-dependent ATPase activity was measured (see below).

Purification of CF_1. CF_1 was isolated and purified from osmotically shocked, chloroform extracted batch cultures (total volume 10 to 12 L) of *D. salina* as previously described [13]. The enzyme was stored as a suspension in 50% saturated ammonium sulfate at 0 to 4°C.

N-ethylmaleimide treatment of soluble CF_1. Aliquots of the ammonium sulfate suspended protein were centrifuged (12000 × *g*; 2 min), and 100 μg of the protein were dissolved in 0.1 ml buffer containing 20 mM Tricine-NaOH (pH 8.0) and 1.0 mM ethylenediamine tetraacetic acid (EDTA). *N*-ethylmaleimide was added, and the mixture was incubated as described in the figure legends. The reaction was quenched by the addition of a fourfold excess of dithiothreitol or excess reagents were removed with or without the addition of dithiothreitol by centrifugation of the mixture through 1.0 ml Sephadex G-50, equilibrated with 20 mM Tricine-NaOH (pH 8.0) and 1.0 mM EDTA [18]. For labeling experiments, the specific activity of [^{3}H]*N*-ethylmaleimide was approx. 34 μCi/μmol, and the concentration of *N*-ethylmaleimide was varied from 0.5 to 2.0 mM.

ATPase assays. The ethanol-induced (20%, v/v), Mg^{2+}-dependent and heat-activated, Ca^{2+}-dependent ATPase activities were measured as previously described [13] at 37°C for 2 min.

Miscellaneous. Chlorophyll [19] and protein [17], using bovine serum albumin as a standard, were measured as previously described. [γ-^{32}P]ATP was synthesized and purified essentially as described by Magnusson et al. [20]. SDS-denaturing polyacrylamide slab gel electrophoresis was performed as described by Laemmli [21]. In order to obtain a good separation between the two largest subunits of the ATPase, 18% acrylamide gels were used, and the electrophoresis was run for 48 h. After staining the gels for protein [22], the bands were excised and the radioactivity determined as described by Senior [23].

Materials. [^{32}P]Inorganic phosphate and [^{3}H]

N-ethylmaleimide were obtained from New England Nuclear. All other reagents were of the finest grade available. Cultures of *D. salina* were kindly provided by Prof. Mordhay Avron, The Weizmann Institute of Science, Rehovot, Israel.

Results and Discussion

Photophosphorylation catalyzed by Dunaliella salina thylakoid membranes. Relatively high rates (approx. 600–800 μmol ATP synthesized/mg Chl/h) of phenazine methosulphate-dependent photophosphorylation can be obtained from crude thylakoid membrane preparations isolated from osmotically shocked cultures of *D. salina* if care is taken to harvest the cells during the log growth phase. This relatively high rate decreases dramatically after cultures enter the stationary growth phase. We have obtained similar results with thylakoid membranes prepared from the unicellular green alga *Chlamydomonas reinhardtii* (Selman-Reimer, S. and Selman, B.R., unpublished results).

McCarty et al. [11] have shown that the treatment of spinach thylakoid membranes with *N*-ethylmaleimide in the light, but not in the dark, results in a partial but permanent inhibition of photophosphorylation. In contrast, as shown in Fig. 1, treatment of *D. salina* thylakoid membranes in the dark with *N*-ethylmaleimide severely inhibits phenazine methosulphate-dependent photophosphorylation. As is the case with spinach thylakoid membranes, *N*-ethylmaleimide inhibition of photophosphorylation is only partial, the maximum extent being about 65%; however, the sensitivity of photophosphorylation catalyzed by *D. salina* thylakoid membranes to inhibition by *N*-ethylmaleimide (under almost identical reaction conditions) is markedly lower, 50% of the maximal inhibition being achieved after a 5.0 min incubation at 30°C in the presence of 50 μM *N*-ethylmaleimide. Treatment of the *D. salina* thylakoid membranes with *N*-ethylmaleimide in the light in the presence of phenazine methosulphate does not enhance the extent of inhibition of photophosphorylation (data not shown).

N-ethylmaleimide inhibition of photophosphorylation catalyzed by *D. salina* thylakoid membranes could be due to one or more of a number of factors, including (i) the inhibition of the electron-transfer chain associated with Photosystem I, (ii) the chemical modification of thylakoid-bound proteins, other than the energy transducing complex, which increases the permeability of the thylakoid membrane to protons, and (iii) the inhibition of the catalytic activity of the energy-transducing complex. It would certainly be con-

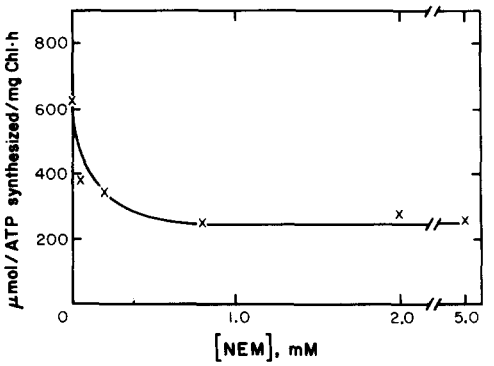

Fig. 1. *N*-ethylmaleimide-inhibition of phenazine methosulphate-dependent photophosphorylation catalyzed by *D. salina* thylakoid membranes. *D. salina* thylakoid membranes were treated for 5 min at 30°C with various concentrations of *N*-ethylmaleimide ([NEM]), as indicated, and assayed for phenazine methosulphate-dependent photophosphorylation as described in Methods and Materials.

TABLE I

N-ETHYLMALEIMIDE INHIBITION OF PHOTOPHOSPHORYLATION AND THE CF_1 ATPase ACTIVITY

D. salina thylakoid membranes were treated with 1.25 mM *N*-ethylmaleimide for 15 min at room temperature as described in Methods and Materials. After the treatment, the membranes were either assayed for phenazine methosulphate-dependent photophosphorylation or extracted with chloroform to solubilize the CF_1. The CF_1 ethanol-induced, Mg^{2+}-dependent ATPase activity was assayed as previously described [13], and the rate was calculated based on the amount of thylakoid membrane material extracted. The numbers in parentheses are percent control.

Membranes	μmol ATP synthesized per mg Chl per h	μmol ATP hydrolyzed per mg Chl per h
Control	725	692
N-ethylmaleimide-treated	320 [44]	375 [54]

ceivable that there are enough differences between the composition of the thylakoid membranes of the halotolerant alga, *Dunaliella,* and vascular plants such that *N*-ethylmaleimide could inhibit either (phenazine methosulphate-dependent) electron transfer or the maintenance of the transmembrane pH gradient. In order to determine if the dark treatment of *D. salina* thylakoid membranes with *N*-ethylmaleimide has a direct effect on the catalytic activity of CF_1, thylakoid membranes were treated with *N*-ethylmaleimide (1.25 mM for 15 min at room temperature), the coupling factor released from the membranes (by chloroform extraction), and the ethanol-induced, Mg^{2+}-dependent ATPase determined. These results were compared to the effect on photophosphorylation of the dark treatment of thylakoid membranes with *N*-ethylmaleimide and are summarized in Table I. Clearly both photophosphorylation and the ATPase activity of the extracted CF_1 are inhibited by the treatment of the thylakoid membranes in the dark with *N*-ethylmaleimide and both are inhibited to about the same extent (55% and 45% inhibition of photophosphorylation and ATPase activity, respectively).

That the inhibition of photophosphorylation is directly related to the chemical modification of thylakoid-bound CF_1 was confirmed by measuring the time courses for the *N*-ethylmaleimide inhibition of photophosphorylation and the ATPase activity of the extracted coupling factor. Thylakoid membranes were treated with 1.25 mM NEM at 0–4°C for various times and then assayed either for photophosphorylation or the Mg^{2+}-dependent ATPase activity of the thylakoid-released coupling factor. Fig. 2 clearly demonstrates that the time-courses for the *N*-ethylmaleimide inhibition of both activities are identical. Thus, we conclude that the *N*-ethylmaleimide inhibition of photophosphorylation is a direct effect of *N*-ethylmaleimide modification of CF_1, a process that does not require a proton-motive-force-induced conformational change in CF_1. This distinguishes the *D. salina* CF_1 from its higher vascular plant counterpart.

N-ethylmaleimide inhibition of the ATPase activity of the solubilized Dunaliella CF_1. The ATPase activity of soluble spinach CF_1 is not sensitive to inhibition by *N*-ethylmaleimide unless the chemical modifier is present during the heat-activation

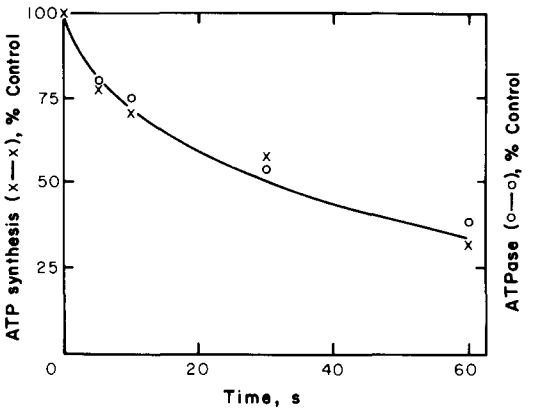

Fig. 2. Time-course for *N*-ethylmaleimide inhibition of phenazine methosulphate-dependent photophosphorylation and CF_1 ATPase activity. *D. salina* thylakoid membranes were treated with 1.25 mM *N*-ethylmaleimide at 0–4°C for the indicated times as described in Methods and Materials. Membranes were then either assayed for phenazine methosulphate-dependent photophosphorylation (× ——— ×, control rate, 210 μmol ATP synthesized per mg Chl per h) or extracted with chloroform to solubilize the CF_1 which was assayed for the ethanol-induced, Mg^{2+}-dependent ATPase [13] (○ ——— ○, control rate, 27 μmol ATP hydrolyzed per mg protein per min).

phase [24]. That this is not the case for the *D. salina* CF_1 is shown in Fig. 3 for an experiment in which the latent enzyme was treated with *N*-ethylmaleimide prior to the determination of the ethanol-induced, Mg^{2+}-dependent ATPase activity. Treatment of the latent enzyme with *N*-ethylmaleimide again results in a partial (approx. 65% maximal) inhibition of the inducible ATPase activity, half maximal inhibition requiring, under these conditions, approx. 0.35 mM *N*-ethylmaleimide. *N*-ethylmaleimide treatment of both the latent and heat-activated coupling factor results in the same extent of inhibition of both the Mg^{2+}- and Ca^{2+}-dependent CF_1 ATPase activities (data not shown). Clearly the *D. salina* CF_1 in its latent state, either when bound to thylakoid membranes or solubilized, must contain an accessible sulfhydryl group that, when modified by *N*-ethylmaleimide, leads to the inhibition of its catalytic activity.

Restoration of the D. salina CF_1 ATPase activity. The above results demonstrate that the ATPase activity of the *D. salina* CF_1 can be markedly, albeit only partially, inhibited by treatment with *N*-ethylmaleimide regardless of whether the treatment is performed in situ (i.e., when CF_1 is bound

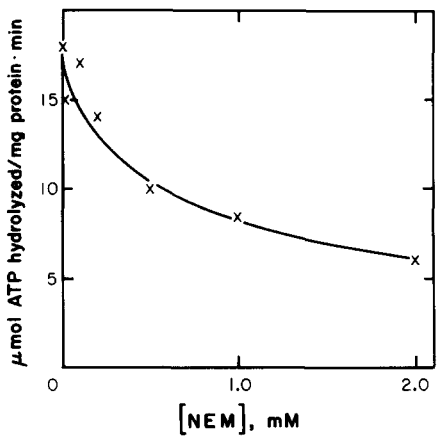

Fig. 3. *N*-ethylmaleimide (NEM) inhibition of the latent ATPase activity of the purified *D. salina* CF_1. The *D. salina* CF_1 was purified, treated with the indicated concentrations of *N*-ethylmaleimide for 30 min at 27°C, and assayed for the ethanol-induced Mg^{2+}-dependent ATPase as described in Methods and Materials.

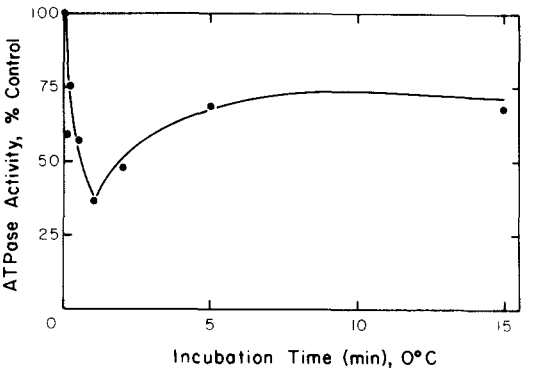

Fig. 4. Extended time-course for the effect of *N*-ethylmaleimide on the ATPase activity of the purified *D. salina* CF_1. The purified *D. salina* CF_1 was treated with 1.25 mM *N*-ethylmaleimide at 0–4°C for the indicated times as described in Methods and Materials and assayed as in Fig. 3.

to thylakoid membranes) or in vitro. The fact that the inhibition is only partial and not affected by the presence of ligands to CF_1 (not shown) strongly suggests that the group(s) modified is (are) not located at the active site of the enzyme. A more compelling argument, however, is the observation that the *N*-ethylmaleimide inhibition of the ATPase activity can also be reversed by *N*-ethylmaleimide. This is demonstrated in Fig. 4, which shows an extended time-course for the effect of 1.25 mM *N*-ethylmaleimide on the ATPase activity of the latent, soluble ATPase when the treatment is performed at 0–4°C. The rapid ($t_{1/2} \approx 15$ s), partial (approx. 65%) inhibition is gradually reversed as the incubation time is extended, such that by 15 min the ATPase activity is only inhibited about 30%, representing a 35% restoration of the original activity.

A similar phenomenon is observed when the inducible ATPase activity is titrated with *N*-ethylmaleimide (at a constant incubation time, 1.0 min, at 4°C) as shown in Fig. 5. Whereas low concentrations of *N*-ethylmaleimide result in a marked inhibition (approx. 60%) of the ATPase ($I_{50} \approx 50$ μM), the inhibition at high concentrations (4–10 mM) *N*-ethylmaleimide is much less severe (approx. 25%), again representing about a 35% restoration of the original activity. Although the ethanol-induced, Mg^{2+}-dependent ATPase activity of the *D. salina* CF_1 is gradually stimulated when the enzyme is incubated in dithiothreitol, which was used to quench the reaction with *N*-ethylmaleimide, the *N*-ethylmaleimide reversal of the *N*-ethylmaleimide inhibition of the ATPase activity is probably not the result of a superimposed dithiothreitol activation. We have obtained similar results to those shown in Fig. 5 when we eliminated the dithiothreitol quench and directly removed excess *N*-ethylmaleimide by rapid gel filtration after incubating enzyme samples (not shown).

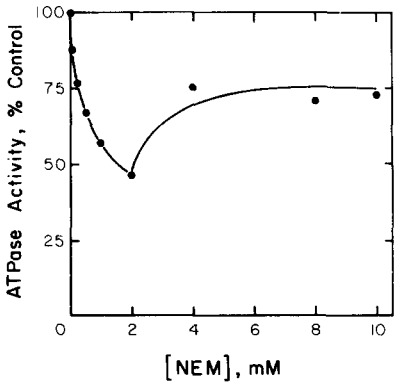

Fig. 5. Concentration curve for the effects of *N*-ethylmaleimide (NEM) on the ATPase activity of the purified *D. salina* CF_1. The purified *D. salina* CF_1 was treated at 0–4°C for 1.0 min with the indicated concentrations of *N*-ethylmaleimide as described in Methods and Materials and assayed as in Fig. 3.

It should be noted that the reversal of the *N*-ethylmaleimide inhibition by *N*-ethylmaleimide occurs whether the latent ATPase is treated in situ or in vitro (not shown). In contrast, however, *N*-ethylmaleimide inhibition of photophosphorylation is not reversed.

At this point in our study, the location for the putative binding sites on the various subunits of CF_1 for *N*-ethylmaleimide that affect the catalytic activity of the enzyme are unclear. Using radiolabeled [^{3}H]*N*-ethylmaleimide, we have observed that 50% inhibition of the *D. salina* CF_1 ATPase activity corresponds to the covalent incorporation of about 5 mol *N*-ethylmaleimide/mol CF_1, but because all three of the largest CF_1 subunits, α, β and γ, covalently bind *N*-ethylmaleimide, it has not yet been possible to determine to which subunit(s) the binding of *N*-ethylmaleimide correlates to the inhibition of activity. Likewise, we have also been unable, as yet, to establish the location of the binding site(s) that, when modified, results in the restoration of activity. Studies designed to clarify these very intriguing issues are currently in progress.

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