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COMMUNICATION

Nucleotide-Protectable Labeling of Sulfhydryl Groups in Subunit I of the ATPase from *Halobacterium saccharovorum*[1]

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A membrane-bound ATPase from the archaebacterium *Halobacterium saccharovorum* is inhibited by *N*-ethylmaleimide in a nucleotide-protectable manner (Stan-Lotter *et al.*, 1991, *Arch. Biochem. Biophys.* 284, 116–119). When the enzyme was incubated with *N*-[^{14}C]ethylmaleimide, the bulk of radioactivity was associated with the 87,000-Da subunit (subunit I). ATP, ADP, or AMP reduced incorporation of the inhibitor. No charge shift of subunit I was detected following labeling with *N*-ethylmaleimide, indicating an electroneutral reaction. The results are consistent with the selective modification of sulfhydryl groups in subunit I at or near the catalytic site and are further evidence of a resemblance between this archaebacterial ATPase and the vacuolar-type ATPases. © 1992 Academic Press, Inc.

Amino acid sequences of the two major ATPase subunits from *Sulfolobus acidocaldarius* (1, 2), *Methanosarcina barkeri* (3), several vacuolar ATPases (V-type ATPases) (4), and the ATP synthases (F-type ATPases), which occur in mitochondrial, chloroplast, and eubacterial membranes, suggest that archaebacterial ATPases are more closely related to V-type than to F-type ATPases (5). On the other hand, archaebacterial and F-type ATPases are immunologically related (6, 7); the ATPase from the archaebacterium *Halobacterium saccharovorum* is inhibited by DCCD[3] and NBD-Cl under conditions which also affect F-type ATPase activity (8, 9); and the ATPase from *M. barkeri* contains amino acid residues believed to be essential for catalytic function of F-type ATPases (3). These observations suggest that archaebacterial ATPases are chimeric in the sense that they possess features of V-type and F-type ATPases (9–11).

A distinguishing property between V- and F-type ATPases is their sensitivity to the thiol reagent, NEM; unlike F-type ATPases, V-type ATPases are inhibited by NEM (12, 13). The ATPase from *H. saccharovorum* is also inhibited by NEM and the presence of nucleotides decreases the extent of inhibition (9). In this Communication we demonstrate that NEM is incorporated into subunit I, that the conditions for modification are similar to those which result in the inhibition of enzyme activity, and that the reaction involves the specific modification of thiol groups.

MATERIALS AND METHODS

The membrane ATPase from *H. saccharovorum* (ATCC 29252) was prepared as previously described (14, 15). The specific activities following purification on DEAE-cellulose and sucrose gradients were 3.1 and 6.4 μmol PO_4 min^{-1} mg^{-1} protein, respectively. Modification of the enzyme with NEM was carried out in 50 mM triethanolamine-HCl/4 M NaCl/2 mM EDTA, pH 7.15, buffer (9). ATPase activity was determined in the presence of 0.01% Triton X-165 with Mn–ATP as substrate (9). SDS gels were run according to the procedure of Laemmli (16). Isoelectric focusing was done in a two-dimensional system described previously (17) with a concentration of 0.5% ampholytes (18). Gels containing isotopes were soaked in Amplify, dried under reduced pressure, and exposed at −70°C to Kodak XAR 5 film. Densitometry of stained gels or autoradiographs was performed with a Hoefer Model GS 300 or LKB Ultroscan XL laser densitometer. The amount of [^{14}C]NEM associated with protein bands was determined by integration of the area under the peaks. Protein was determined by the method of Bradford (19) with γ-globulin as standard. Bio-Rad protein assay reagent, γ-globulin, and SDS were obtained from Bio-Rad Laboratories. ATP, ADP, AMP, and NEM were from the Sigma Chemical Co. [^{14}C]NEM (specific activity 7 mCi/mmol) and Amplify were from Amersham.

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[3] Abbreviations used: SDS, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DCCD, *N,N'*-dicylcohexylcarbodiimide.

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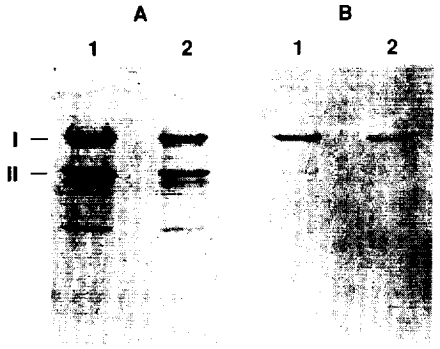

FIG. 1. Time-dependent incorporation of [^{14}C]NEM into subunit I. Halobacterial enzyme (DEAE fraction) was treated with 1 mM [^{14}C]NEM for 3 h (lane 2) and 6 h (lane 1). Following removal of excess reagent, subunits were separated on an SDS gel. Acrylamide concentration was 12%. Gels were stained with Coomassie blue (A) or exposed to X-ray film (B). The major subunits I and II are indicated. Lane 1, 26 μg; lane 2, 22 μg.

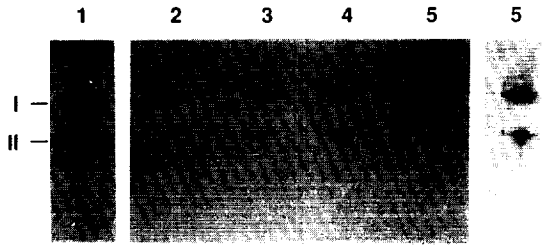

FIG. 2. Effect of nucleotides on the incorporation of [^{14}C]NEM. Prior to reaction with [^{14}C]NEM, the halobacterial enzyme (purified on a sucrose gradient) was incubated for 25 min with 5 mM of the following: AMP, lane 2; ADP, lane 3; ATP, lane 4; no nucleotide, lane 5. Following reaction with NEM for 5 h, the procedure was as described in the legend to Fig. 1. (left and right) Coomassie-stained gels; (middle) gel exposed to X-ray film. Lane 1, untreated enzyme, 9.5 μg; lanes 2–5, approximately 9.5 μg each.

RESULTS

Figure 1 shows the results of an experiment in which the ATPase from *H. saccharovorum* was incubated in the presence of [^{14}C]NEM. The bulk of the radioactivity was associated with subunit I (M_r 87,000) and, as was the case with inhibition of hydrolytic activity (9), the amount incorporated increased with the time of incubation (Fig. 1B, lanes 1 and 2). Densitometric scans indicated that the incorporation of [^{14}C]NEM into subunit I was 28% greater after 6 h than after 3 h. The radioactivity in subunit I, again estimated by densitometric scanning (lane 1, Fig. 1B), was more than 75% of the total incorporated NEM. Some radioactivity was present in subunit II (M_r 60,000) and in a faster migrating protein (M_r 49,000). The amount of [^{14}C]NEM incorporated into subunit II or the faster moving component did not increase with time, suggesting that the site of NEM inhibition was subunit I. The incorporation of [^{14}C]NEM into subunit I was reduced, as was the inhibition, when the enzyme was preincubated with nucleotides prior to treatment with NEM. ADP and ATP (Fig. 2, lanes 3 and 4, respectively) were the most effective, reducing the relative label by 62 and 65%, respectively, whereas AMP (Fig. 2, lane 2) was the least effective, causing a reduction of about 42%. The hydrolytic activities (as % of the uninhibited enzyme) associated with the samples used in Fig. 2 were 57% (lane 2, AMP); 80% (lane 3, ADP); 83% (lane 4, ATP); and 29% (lane 5, no nucleotides). The enzyme shown in Fig. 2 was purified on a sucrose gradient (20) which removed a number of contaminating proteins. Thus, the extensive time required to demonstrate inhibition by NEM did not lead to any significant degradation of subunits I or II since no additional components were observed (compare Fig. 2, lanes 1 and 5).

The prolonged incubation times and the near neutral conditions employed to achieve maximum inhibition and incorporation of NEM raised the possibility that the inhibitor could have reacted with groups other than thiols, most notably amino groups. A relatively simple experiment based on the following considerations ruled out the participation of amino groups. The reaction between NEM and thiols is electroneutral, whereas a reaction between NEM and amino groups results in the appearance of protein species with one additional net negative charge per modified group. Such species are resolvable on isoelectric focusing gels [see for example Ref. (21)]. The result of a typical experiment in which the enzyme was subjected to isoelectric focusing following labeling with [^{14}C]NEM is shown in Fig. 3. Subunit I migrated as a double band, indicating charge heterogeneity, as had previously been observed (18). However, no additional anodically migrating radioactive bands were detected (Fig. 3, lane 1) as would have been expected had a reaction taken place between an amino group and NEM. We take this as confirmation that the reaction between the ATPase and NEM is electroneutral and therefore involves a thiol group.

DISCUSSION

Although the α and β subunits of F-type ATPases contain cysteinyl residues (22), thiol groups are not involved

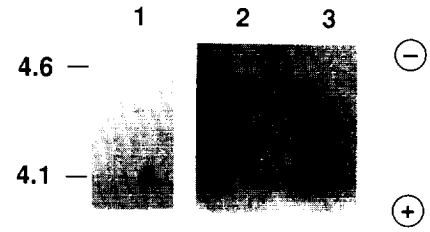

FIG. 3. Isoelectric focusing of subunit I. The enzyme was treated with [^{14}C]NEM. Subunit I was separated on an isoelectric focusing gel as described in (20). The gel was stained with Coomassie blue (lanes 2 and 3) or exposed to X-ray film (lane 1). The approximate pH range is indicated to the left. Lanes 1 and 2, 8 μg of subunit I from [^{14}C]NEM-treated enzyme; lane 3, 10 μg of subunit I from untreated enzyme.

in catalysis. In the case of the ATPase from *Escherichia coli*, either the subunits do not react with sulfhydryl reagents or,if they do, hydrolytic activity is marginally affected (23). V-type ATPases are characteristically inhibited by low concentrations of NEM (12, 13). The inhibitor predominantly labels the largest subunit (subunit A) and the inhibition is relieved by nucleotides. This has lead to the suggestion that subunit A is the catalytic one and thiols are associated with the catalytic site [for review see (24)].

The characteristics of the incorporation of NEM into the ATPase from *H. saccharovorum* were similar to those leading to the inhibition of enzyme activity [see Ref. (9)]. Both exhibited a marked time dependence and both processes were antagonized by the presence of adenine nucleotides. Protection by ATP and ADP was consistent with the observation that the latter is a competitive inhibitor with respect to ATP (14). While AMP is neither a substrate nor a product of ATP hydrolysis (14), it inhibited the halobacterial ATPase (Stan-Lotter and Hochstein, unpublished). AMP also inhibits the V-type ATPase from oat tonoplasts (25), which is another indication of the close relationship between V-type APTase and the halobacterial ATPase. The incorporation of NEM into subunit I was electroneutral as would be predicted if the inhibitor reacted with cysteinyl residues. Nonspecific labeling (other than subunit I) was less than 25% of the total NEM associated with the halobacterial enzyme. Our results suggested that cysteinyl residues in subunit I, which are readily accessible to modification by NEM, may be located at or near the substrate binding site. Consistent with this notion is the presence of a nucleotide binding site, deduced from amino acid sequence data, in the largest subunit from the ATPase from *H. salinarium* (26).

Nucleotide analogues bind to both of the major subunits from the F-type ATPases (27) and the ATPase of *S. acidocaldarius* (10). NBD-Cl, a nucleotide analogue, also inhibits the ATPase from *H. saccharovorum* in a nucleotide protectable manner. In this case, the bulk of the NBD-Cl is associated with subunit II even though the conditions of inhibition are similar to those used to demonstrate NEM inhibition (9). The inhibition of the halobacterial ATPase by DCCD is accompanied by the binding of this inhibitor to subunit II (8). The conditions are analogous to those which result in the inhibition of F-type ATPase activity where DCCD is bound to the β subunit which contains the catalytic site (28). Thus, it remains to be established which of the halobacterial subunits contains the catalytic site and, if both subunits do indeed possess nucleotide binding sites, how such sites contribute toward catalytic activity.

In summary, the effect of NEM on the halobacterial ATPase with respect to the time dependence of inhibition, the antagonistic action of adenine nucleotides, and the site of inhibition indicates that thiol groups are essential for enzyme activity and is a further confirmation that the ATPase from *H. saccharovorum* possesses features which are characteristic of V-type ATPases.

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