Vol. 81, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1978 Pages 439-447

ACCESSIBILITY OF THE α CHAINS IN MEMBRANE-BOUND AND SOLUBILIZED BACTERIAL ATPase TO CHYMOTRYPTIC CLEAVAGE

Richard M. Leimgruber, Charlotte Jensen and Adolph Abrams

Department of Biochemistry

University of Colorado School of Medicine

4200 East Ninth Avenue, Denver, Colorado 80262

Received January 23,1978

SUMMARY: Limited chymotryptic cleavage of the α subunits in the solubilized ATPase from Streptococcus faecalis is accompanied by loss of membrane binding capacity (Abrams, A., Morris, D., Jensen, C. (1976) Biochem. 15, 5560). To obtain evidence that the α chains might function directly in membrane attachment we compared the effect of chymotrypsin on the soluble and membrane-bound enzyme. Using a low level of chymotrypsin the soluble ATPase was quantitatively converted to a catalytically active form in which the 55000 dalton α chains were shortened by approximately 2000 daltons. However, at 80 fold higher levels of chymotrypsin the ATPase in a reconstituted ATPase-membrane complex was completely unaffected. Protection from chymotryptic attack appeared to be membrane specific since the soluble ATPase was not protected by addition of massive amounts of bovine serum albumin. The total and specific immunity to chymotrypsin conferred by membrane binding indicates that chymotrypsin-sensitive α chain "tails" are closely associated with or buried in the membrane. These findings support the view that the α chains are involved directly in membrane attachment.

INTRODUCTION: In the fermentative lactic acid organism, Streptococcus faecalis, the energized transport of various solutes depends solely on ATP hydrolysis catalyzed by an ATPase attached to the plasma membrane (1,2,3). There is considerable evidence that the energy coupling mechanism operates according to the chemiosmotic theory of Mitchell (4), whereby ATP hydrolysis generates a transmembrane protonmotive force which is used secondarily for the performance of osmotic work. It is believed that the protonmotive force arises from an electrogenic extrusion of protons through a specialized membrane "proton channel" which is physically capped by the ATPase (5,6,7). It has been suggested also that conformational changes in the ATPase, associated with ATP binding and hydrolysis are involved in the coupling mechanism (8). However, the molecular mechanism of the energy transduction process is not clear.

0006-291X/78/0812-0439$01,00/0

Many of the structural and catalytic properties of the solubilized S. faecalis ATPase have been fairly well characterized (2,3,9). The enzyme is very similar to the coupling factor ATPases in aerobic bacteria, chloroplasts and mitochondria which mediate oxidative phosphorylation as well as ATP-linked energy functions (10). It is a 385000 dalton multimeric protein (11) that contains tightly bound non-exchangeable nucleotide, ATP and ADP (12,13) and consists of 5 types of subunits with the following approx. mol. wts.: α=55K, β=50K, γ=37K, δ=20K and ε=12K (14). The most likely subunit stoichiometry appears to be $\alpha_3\beta_3\gamma\delta\varepsilon$ (9). Little is known about the structural basis for the association of the enzyme with the membrane. Multivalent cations, e.g. Mg^{2+}, are very likely involved in binding since release of the enzyme can be achieved by washing the ATPase-membrane complex in low ionic strength buffer provided that multivalent cations are absent (15); moreover, reattachment of enzyme to depleted membranes requires the addition of multivalent cations (16). Also needed for binding is the δ subunit (14) which corresponds to the attachment factor, nectin (17). Mg^{2+} ions may also be required to maintain the linkage between the δ chain and the core enzyme (14). That the δ subunit is needed for membrane binding has been established also for the coupling factor ATPase in E. coli (18) and in chloroplasts (19).

A recent study of the S. faecalis ATPase has implicated a portion of the α chain in membrane binding (9). It was found that selective removal of an approx. 2000 dalton terminal piece of the α chains by limited chymotrypsin treatment, yielded a modified enzyme which was fully active but failed to rebind to depleted membranes. A similar finding was subsequently reported by Ritz and Brodie (20) in a study of the effect of trypsin on the soluble ATPase of Mycobacterium phlei. The ATPase α chains of a Micrococcus species also have a protease sensitive terminal region (21). Thus it seems that this structural feature might be common to all the proton translocating ATPases at least in bacteria. Our observations led us to suggest that the chymotrypsin-sensitive terminal segment of the α chains were unfolded tail-like regions which could

interact directly with complementary regions in the membrane receptor site. It is reasonable to expect that such a direct interaction with the membrane would render the α chain "tails" at least partially if not totally inaccessible to chymotryptic cleavage. To test this possibility we have compared the effect of chymotrypsin on the soluble ATPase and a reconstituted ATPase-membrane complex.

METHODS: Preparation of Purified Soluble ATPase and Depleted Membranes. Streptococcus faecalis cells (ATCC 9790) were harvested from 10 ℓ. of growth media containing 1% bactotryptone, 0.5% yeast extract, 1% glucose, 100 mM KCl and 4 mM K_2HPO_4. The cells were washed and incubated for 60 min. at 38°C in 100 ml of 20 mM Tris·Cl, pH 7.5, 10 mM KCl, and 1% glucose, and then coverted to protoplasts by treatment with lysozyme in 0.4 M sucrose. The protoplasts were lysed by osmotic shock to produce membrane ghosts (1,22). To solubilize the enzyme, the membranes were washed repeatedly with low ionic strength Tris Cl, pH 7.5 (14,22). The depleted membranes were lyophilized and saved for subsequent reconstitution of the ATPase-membrane complex. The crude washes containing the ATPase were pooled, adjusted to 20 mM Tris·Cl (pH 7.5), 10 mM $MgCl_2$, 20% glycerol, and then dialyzed against the same buffer at 4°C overnight. The enzyme was then adsorbed onto a Bio-Gel A DEAE-cellulose column (Bio-Rad, 4.5 cm x 20 cm) and eluted with 400 ml of a 0.1 - 0.3 M KCl gradient in 20 mM Tris·Cl (pH 7.5), 10 mM $MgCl_2$, 20% glycerol, 0.5 mM ATP. The enzyme was then concentrated by pressure ultrafiltration (Amicon PM-30) to a volume of 1 ml. Finally, the ATPase was purified by zonal sedimentation on a linear 4-16% sucrose (ultra-pure, Schwarz-Mann) gradient in 20 mM Tris·Cl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM ATP (22). ATPase assays were performed as previously described (1). One unit of ATPase activity is the amount of enzyme necessary to yield one μmole of P_i per min. at 38°C.

Preparation of Reconstituted ATPase-Membrane Complex. The procedure was similar to that described previously (16,22). Lyophilized depleted membranes were rehydrated in 100 mM Tris·Cl (pH 7.5), 20 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride and pelleted at 78,000 x g for 20 min. The membrane pellet was then resuspended in a solution of the purified soluble ATPase preparation in 20 mM Tris·Cl (pH 7.5), 10 mM $MgCl_2$ in the ratio of 100 units ATPase per mg of depleted membrane, dry weight. Following incubation for 1 hour at 38°C, the resulting reconstituted ATPase-membrane complex was centrifuged at 78,000 x g for 15 min. The reconstituted complex was resuspended in 20 mM Tris·Cl (pH 7.5), 10 mM $MgCl_2$ at 3.0 - 3.5 units ATPase per ml.

Chymotrypsin Treatment of the Soluble and Membrane-Bound ATPase. The soluble ATPase or the reconstituted membrane bound ATPase in 20 mM Tris·Cl (pH 7.5), 10 mM $MgCl_2$ was treated with bovine pancreatic α-chymotrypsin (3x crystallized, Sigma) for 20 min. at 38° at the levels indicated in the figure legends (9). The digestion was terminated by the addition of phenylmethylsulfonylfluoride (PMSF), using 15 times the amount of chymotrypsin by weight. PMSF had no significant effect on the catalytic or structural properties of the enzyme (9).

Solubilization of ATPase after Chymotrypsin Treatment of the Reconstituted Complex. Following treatment of the reconstituted membrane-bound ATPase preparation with chymotrypsin the enzyme was solubilized by repeatedly washing the membranes with Mg^{2+}-free dilute Tris buffers essentially as previously described (15). First the suspension was centrifuged at 105,000 x g for 10 min.

The pellet was then resuspended and centrifuged repeatedly, twice in 33 mM Tris·Cl (pH 7.5) and four to six times more in 1 mM Tris·Cl (pH 7.5) buffer. The solubilized ATPase generally appeared in the 3rd to 5th wash.

Polyacrylamide Gel Electrophoresis Under Non-Dissociating Conditions. Analyses of samples under nondissociating conditions were performed with 3 mm vertical slab gels containing 5% polyacrylamide in Tris·glycine, pH 8.5, and 10 mM $MgSO_4$ (16). Protein was detected by staining with 0.05% Coomassie blue in methanol:acetic acid:water, 5:1:5, and destaining with the same solvent. ATPase activity in unstained gels was detected by application of the Fiske-Subbarow reagents for the detection of P_i after soaking the gels in Mg ATP according to the modified procedure of Abrams and Baron (23,9).

Subunit Analysis of ATPase Bands Excised from Polyacrylamide Gels. For subunit analysis we used electrophoretically purified ATPase. The native ATPase band resolved by electrophoresis under nondissociating conditions on 3 mm slab gels and stained with Coomassie blue (see above) was excised from the gel for further electrophoretic analysis under dissociating conditions as previously described (12). For this purpose the gel slice was first dehydrated by shaking in methanol for approximately 1/2 hour followed by shaking for an additional 1/2 hour in a 1:1 methanol-ethanol mixture. The dry gel slice was then rehydrated by soaking it overnight in a minimal volume (150-200 µl) of 5% sodium dodecyl sulfate, 10% mercaptoethanol, 1.0 M phosphate buffer (pH 7.0) to dissociate the protein into its subunits within the gel slice. Since the entire dissociating solution is absorbed by the dehydrated gel slice, all the protein is retained. The treated gel slice was then placed into a slot of a 6 mm polyacrylamide vertical gel slab containing 12% polyacrylamide, 0.1 M phosphate (pH 7.0), 0.1% sodium dodecyl sulfate and 1 mM EDTA for electrophoretic analysis of the subunits.

EXPERIMENTAL AND RESULTS: Effect of chymotrypsin on the soluble ATPase. The soluble form ot the ATPase was incubated with 0.6 µg chymotrypsin per unit of ATPase corresponding to a chymotrypsin:total protein ratio of 1:66. Fig. 1 illustrates the electrophoretic pattern obtained when the treated and untreated ATPase samples were run on gel slabs under non-denaturing conditions and stained for both protein and for catalytic activity. The patterns show that the soluble ATPase was converted completely to a form which retained catalytic activity and migrated about 15% faster than the native enzyme. (Fig. 1).

Electrophoretic analysis of the native and chymotrypsin-treated soluble ATPase under dissociating conditions in gel slabs containing sodium dodecyl sulfate is illustrated in Fig. 2. The amount of enzyme used for the analyses was sufficient to visualize only the major subunits, α and β. The pattern shows that all the α chains (mol. wt. ≈ 55,000) in the chymotrypsin treated ATPase were cleaved to yield chains with a mol. wt. ≈ 53,000 (Fig. 2). There was no discernible effect on the β chains. In a previous study it was shown

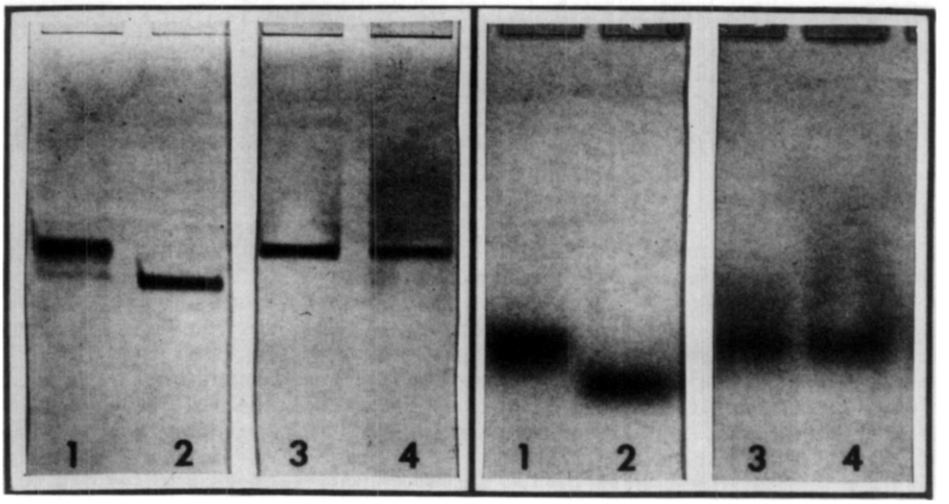

Fig. 1. Electrophoresis of the ATPase under non-dissociating conditions after chymotrypsin treatment of the soluble and membrane-bound enzyme. Left: Gel stained for protein. Right: Gel stained for ATPase activity. 1. Untreated soluble ATPase. 2. Soluble ATPase treated with chymotrypsin (0.6 μg/unit; chymotrypsin:total protein = 1:66). 3. Untreated membrane-bound ATPase. 4. Membrane-bound ATPase treated with chymotrypsin (50 μg/unit ATPase; chymotrypsin:total protein = 1:100). For the analysis the membrane-bound ATPase was solubilized as described under Methods; 0.1 unit (4 μg protein) was applied to the gel.

that the S. faecalis soluble ATPase that was modified by limited chymotryptic digestion as described above failed to reattach to ATPase-depleted membrane (9).

Effect of Chymotrypsin on the Membrane-Bound ATPase. To determine if the α chains were susceptible to cleavage when the ATPase was membrane-bound we incubated a reconstituted ATPase-membrane complex (see Methods) with chymotrypsin. The incubation conditions were the same as those employed to treat the soluble form of the enzyme, but the level of chymotrypsin was increased 80-fold to compensate for the greater amount of total protein in the ATPase-membrane complex. Thus, for the treatment of the membrane-bound ATPase we used 50 μg chymotrypsin per unit of membrane-bound ATP corresponding to a chymotrypsin:total protein ratio of 1:100. Following chymotrypsin treatment the ATPase was solubilized by repeated washing of the membrane complex (see Methods) and then analyzed electrophoretically under non-denaturing and denaturing conditions alongside

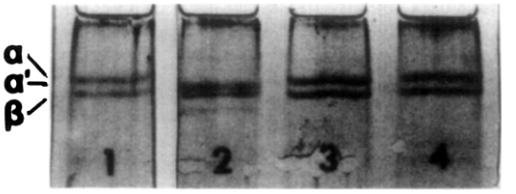

Fig. 2. Subunit analysis of the ATPase in SDS-gels after chymotrypsin treatment of the soluble and membrane-bound enzyme. 1. Untreated soluble ATPase. 2. Soluble ATPase treated with chymotrypsin (0.6 μg) unit chymotrypsin: total protein = 1:66). 3. Untreated membrane-bound ATPase. 4. Membrane-bound ATPase treated with chymotrypsin (50 μg/unit; chymotrypsin:total protein = 1:100). For electrophoretic analysis the membrane-bound ATPase was solubilized and purified electrophoretically (see Methods). Because of the small amount of protein only the α and β chains could be visualized. The α'band refers to the chymotrypsin modified α chains (9).

the chymotrypsin-treated soluble ATPase (Fig. 1 and Fig. 2). The results show that the ATPase stripped from the chymotrypsin-treated membrane complex remained intact. There was no visible change in the catalytic activity or in the electrophoretic mobility under non-denaturing conditions, (Fig. 1),and there was no cleavage of the alpha chains observable in the SDS gel analysis (Fig. 2).

It is conceivable that the protection of the α chains conferred by membrane binding might be due simply to a non-specific interference by the relatively massive amount of other membrane protein. However we obtained evidence that such a non-specific protective effect is unlikely. As illustrated in Fig. 3, we found that the soluble form of the ATPase was not protected at all from chymotryptic cleavage by the addition of massive amounts of bovine serum albumin such that the chymotrypsin:total protein ratio was 1:2000. By contrast membrane binding fully protected the ATPase at a chymotrypsin:protein ratio of 1:100 (Fig. 1 and Fig. 2).

DISCUSSION: No specific function of the α subunits in the energy transducing ATPases of bacteria, chloroplasts and mitochondria has as yet been established. Through the use of limited chymotryptic cleavage as a probe of structure-function relationships we have obtained two kinds of evidence indicating that the α chains in the ATPase of S. faecalis are involved in membrane attachment. As

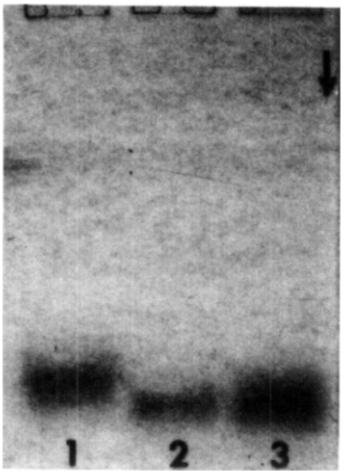

Fig. 3. Electrophoresis of the soluble ATPase under non-dissociating conditions after chymotrypsin treatment in the presence of bovine serum albumin. 1. Untreated ATPase. 2. ATPase treated with chymotrypsin (2 μg/unit, chymotrypsin:total protein = 1:20). 3. ATPase mixed with bovine serum albumin (14 mg/ml) treated with chymotrypsin (2 μg/unit; chymotrypsin:total protein = 1:2000). The gel was stained for ATPase activity (9).

reported earlier (9), limited chymotryptic treatment of the soluble ATPase selectively removed an approx. 2000 dalton fragment from the 55000 dalton α chain. Although the modified enzyme retained catalytic activity, it could not rebind to depleted membranes as does the native enzyme, thus implicating chymotrypsin-sensitive α chain "tails", directly or indirectly, in the binding mechanism. Here, we have shown that in a reconstituted ATPase-membrane complex the α chain "tails" become totally immune to chymotryptic attack (Fig. 1 and Fig. 2). Despite treatment of the ATPase-membrane complex with relatively high levels of chymotrypsin per unit of ATPase, the α chains were unaffected. By contrast, a much lower level of the protease per unit of ATPase readily cleaved the α chains in the solubilized form of the enzyme. In the treatment of both the soluble and the membrane-bound forms of the ATPase essentially the same chymotrypsin:total protein ratio was used. The protective effect conferred by the membrane was not only complete but seemed to be specific as well, since massive amounts of bovine serum albumin added to the soluble ATPase failed to

protect the α chains from chymotryptic attack (Fig. 3). In this regard it should be noted that a specific type of rebinding to the depleted membrane is to be expected since the reconstituted ATPase-membrane complex is sensitive to the energy transfer inhibitor, dicyclohexylcarbodiimide, which is directed at the proton channel (24,7).

In earlier findings limited cleavage of the α chain in the soluble S. faecalis ATPase was associated with loss of its capacity to rebind to depleted membrane (9) while the experiments presented here shows that rebinding to the membrane renders the α chain "tails" inaccessible to chymotryptic cleavage indicating that the α chain "tails" are close to, or possibly buried in, the membrane. Taken together these observations provide strong evidence that the ATPase α chains are involved in membrane attachment through direct association with the membrane receptor site.

ACKNOWLEDGEMENT: This work was supported by a grant from the National Institute of General Medical Sciences.

REFERENCES

1. Abrams, A., McNamara, P., and Johnson, F.B. (1960) J. Biol. Chem., 235, 3659.
2. Abrams, A. and Smith, J.B. (1974) The Enzymes, (Boyer, P.D., ed.) Vol. 10, pp. 395-429, Academic Press, New York.
3. Abrams, A. (1976) The Enzymes of Biological Membranes, (Martonosi, A., ed.) Vol. 3, pp. 57-73, Plenum Publishing Corp., New York.
4. Mitchell, P. (1976) Biochem. Soc. Trans. 4, 399.
5. Mitchell, P. (1973) FEBS Lett. 33, 267.
6. Altendorf, L., Harold, F., and Simoni, R. (1974) J. Biol. Chem., 249, 4587.
7. Simoni, R. and Postma, W. (1975) Ann. Rev. Biochem., 44, 523.
8. Boyer, P.D. (1977) Ann. Rev. Biochem., 46, 957.
9. Abrams, A., Morris, D., and Jensen, C. (1976) Biochemistry, 15, 5560.
10. Haddock, B. and Jones, C.W. (1977) Bacteriological Reviews, 41, 47.
11. Schnebli, H., Vatter, A., and Abrams, A. (1970) J. Biol. Chem., 245, 1122.
12. Abrams, A., Nolan, E.A., Jensen, C., and Smith, J.B. (1973) Biochem. Biophys. Res. Commun., 55, 22.
13. Abrams, A., Jensen, C., and Morris, D. (1975) J. Supramol. Struct., 3, 261.
14. Abrams, A., Jensen, C., and Morris, D. (1976) Biochem. Biophys. Res. Commun., 69, 804.
15. Abrams, A. (1965) J. Biol. Chem., 240, 3675.
16. Abrams, A. and Baron, C. (1968) Biochemistry, 7, 501.
17. Baron, C., and Abrams, A. (1971) J. Biol. Chem., 246, 1542.
18. Smith, J.B., and P. Sternweiss (1975) Biochem. Biophys. Res. Commun., 62, 764.
19. Younis, H.M., Winget, G.D., and Racker, E. (1977) J. Biol. Chem., 252, 1814.
20. Ritz, E.J., and Brodie, A.F. (1977) Biochem. Biophys. Res. Commun., 75, 933.
21. Hoeckel, M., Hulla, F.W., Risi, S., and Dose, K. (1976) Biochim. Biophys. Acta, 429, 1020.

22. Abrams, A., Baron, C., and Schnebli, H. (1974) Methods in Enzymology, Vol. 32 (Fleischer, S. and Packer, L., eds.), Part B, pp. 428-439, Academic Press, New York.
23. Abrams, A. and Baron, C. (1967) Biochemistry, 6, 225.
24. Abrams, A., Smith, J.B., and Baron, C. (1972) J. Biol. Chem., 247, 1484.