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Chymotryptic Conversion of Bacterial Membrane ATPase to an Active Form with Modified α Chains and Defective Membrane Binding Properties†

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ABSTRACT: The effect of chymotrypsin on the structure, catalytic activity, and membrane binding properties of the energy transducing ATPase in *Streptococcus faecalis* was examined. Chymotrypsin caused a limited cleavage of the solubilized ATPase producing a fully active protein which migrated as a single faster moving band in gel electrophoresis. The modified ATPase, designated CHY-ATPase, was relatively resistant to further chymotryptic alteration. It retained the full complement of tightly bound nonexchangeable nucleotide present in the native ATPase. The CHY-ATPase contained modified α chains, designated α', which were about 2000 daltons smaller than the native 55 000-dalton α chains. However, no alterations in the other subunits, β, γ, δ, or ϵ, were detected. It appears that the subunit composition of the native ATPase, $\alpha_3\beta_3\,\gamma\,\delta\,\epsilon$, was changed to $\alpha'_3\beta_3\,\gamma\,\delta\,\epsilon$. In contrast to the native ATPase, the CHY-ATPase failed completely to reattach to depleted membranes. We have concluded that short chymotrypsin-sensitive α chain "tails" protrude from the ATPase surface and that these peptide segments are needed for membrane attachment. The δ chain was also shown to be required for attachment, confirming previous work (Abrams, A., Morris, D., and Jensen, C. (1976), *Biochem. Biophys. Res. Commun. 69,* 804). This suggests that the three α chains and the δ chain act together as a device to ensure firm association of the ATPase with the membrane.

The membrane-bound ATPase in the fermentative organism, *Streptococcus faecalis,* serves to link solute accumulation to the hydrolysis of glycolytically generated ATP (Harold et al., 1969; Abrams et al., 1972; Smith and Abrams, 1973). It is believed that the ATPase mediates proton extrusion through the membrane thereby generating an electrochemical potential which then drives specific transmembrane solute movements (Harold, 1972; Simoni and Postma, 1975). The molecular basis of this chemiosmotic coupling mechanism (Mitchell, 1966) is poorly understood at present. To arrive at a better understanding of this energy transducing system, we have sought for a number of years to characterize the structure of the ATPase and the membrane components with which it is associated. Some years ago it was shown that the solubilized *S. faecalis* ATPase is a 385 000-dalton oligomeric protein made up of nonidentical subunits (Abrams, 1965; Abrams and Baron, 1967; Schnebli and Abrams, 1970; Schnebli et al., 1970). A recent assessment of the subunit composition indicates that the enzyme consists of five subunit types with approximate mo-

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lecular weights as follow: α = 55 000–60 000, β = 50 000–55 000, γ = 35 000, δ = 20 000, and ϵ = 12 000 (Abrams et al., 1976). In this regard the *S. faecalis* ATPase appears to be structurally homologous to the energy transducing ATPases found in other bacteria (Abrams and Smith, 1974; Abrams et al., 1975) and in mitochondria and chloroplasts (Senior, 1973; Caterall et al., 1973; Penefsky, 1974).

The *S. faecalis* ATPase rebinds readily to depleted membranes through an interaction that requires Mg^{2+} or other multivalent cations (Abrams, 1965; Abrams and Baron, 1968). Significantly the reconstituted complex like the original ATPase–membrane complex is highly sensitive to inhibition by dicyclohexylcarbodiimide, while the solubilized ATPase is insensitive to this agent. On this basis as well as from other evidence, it is believed that the original binding sites in the membrane are reoccupied when the ATPase rebinds to depleted membranes (Harold et al., 1969; Abrams et al., 1972; Smith and Abrams, 1973; Abrams and Baron, 1968). In further studies of rebinding, Baron and Abrams (1971) found that a protein factor associated with the enzyme was required. This factor, called *nectin,* appeared to be linked to the enzyme by Mg^{2+} ions since it dissociated from the enzyme during gel filtration or DEAE[1]-cellulose chromatography if Mg^{2+} ions were absent (Baron and Abrams, 1971; Abrams et al., 1974). The molecular weight of nectin determined by gel filtration under nondissociating conditions was about 37 000. Recently Abrams et al. (1976) reported that Mg^{2+} ions act as cross-links between the δ subunit (20 000 daltons by sodium dodecyl sulfate gel electrophoresis) and the remainder of the enzyme. Furthermore, a δ-free enzyme preparation obtained by preparative gel electrophoresis in the absence of Mg^{2+} ions failed to reattach to membranes. Therefore it appeared that Mg^{2+} links the δ chain to the enzyme and that the δ chain in turn anchors the enzyme to the membrane. These findings suggested that nectin is functionally equivalent to δ and that when isolated it exists as a dimeric form of the δ chain (Abrams et al., 1976). In the *Escherichia coli* ATPase also, the δ chain has been identified as an attachment factor (Bragg et al., 1973; Futai et al., 1974; Smith and Sternweiss, 1975), although in this case its association with the enzyme through Mg^{2+} bridges has not as yet been demonstrated. From the information presently available, the subunit composition and arrangement of the soluble *S. faecalis* ATPase were formulated as: $\alpha_3\beta_3\,\delta\,\epsilon$-$(Mg)_n{}^{2+}$-$\delta$ (Abrams et al., 1976). The same subunit stoichiometry has been suggested for the ATPase of *E. coli* (Bragg and Hou, 1975) and mitochondria (Catterall et al., 1973) but other investigators have offered evidence against it (Senior, 1975; Vogel and Steinhart, 1976). The *S. faecalis* ATPase as isolated also contains some tightly bound nonexchangeable ATP, ADP, and P_i but the location and function of these ligands are not yet known (Abrams and Nolan, 1972; Abrams et al., 1973, 1975). Extrapolating from studies of the chloroplast ATPase (Deters et al., 1975) it is probable that the β subunit contains the catalytic site. As yet, however, the functional role of the subunits α, γ, and ϵ has not been established in either the *S. faecalis* ATPase or the related energy transducing ATPases.

In the work described in this paper we have used chymotrypsin to probe the structure and function relationships in the *S. faecalis* ATPase. We have examined particularly the effect of chymotryptic action on the catalytic activity, the tightly bound nucleotide ligands, the subunit structure, and the membrane binding properties of the enzyme. As will be seen, chymotrypsin under appropriate conditions produces a highly selective and limited cleavage of the α chains without causing any loss of catalytic activity or nucleotide ligands.The catalytically active chymotrypsin-modified ATPase, which we will refer to as CHY-ATPase, is easily recognized as an altered protein by polyacrylamide gel electrophoresis. Interestingly, the CHY-ATPase fails completely to reattach to depleted membranes. The results thus point to a segment of the α chains as playing a critical role in the attachment of the ATPase to the membrane.

Methods

Preparation of Solubilized ATPase. S. faecalis cells (ATCC 9790) were harvested from 30 l. of growth medium, converted to protoplasts with lysozyme and then lysed by osmotic shock to produce membrane ghosts (Abrams et al., 1960). The bound enzyme was solubilized by an aqueous wash procedure (Abrams, 1965; Abrams et al., 1974). The depleted membranes were lyophilized and saved for subsequent assays of reattachment of the solubilized enzyme (Abrams and Baron, 1968; Baron and Abrams, 1971; Abrams et al., 1974). The enzyme was assayed as described by Abrams et al. (1960). One unit is the amount of enzyme which produces 1 μmol of P_i per min at 38 °C.

Purification of the ATPase. The crude solubilized ATPase in 20 mM Tris-Cl (pH 7.5)–10 mM $MgCl_2$ was fractionated essentially as described by Schnebli and Abrams (1970), except that a heating step at 80 °C was omitted and 10% glycerol was added to help stabilize the enzyme. Some preparations were purified further by means of preparative slab gel electrophoresis as described previously (Abrams et al., 1976). To obtain the gel purified ATPase containing all five subunit types, 2 mM Mg^{2+} was introduced into the gel and the buffer compartments. To obtain a δ-free ATPase, Mg^{2+} ions were omitted from the preparative gel system.

Polyacrylamide Gel Electrophoresis. Analyses under nondissociating conditions were carried out with 5% polyacrylamide 3-mm slab gels in Tris-glycine buffer, pH 8. Protein was detected by staining with Coomassie blue in CH_3OH–acetic acid–H_2O (5:1:5) and destaining in the same solvent. ATPase activity in the gel was detected by a modified version of the method of Abrams and Baron (1967) which makes use of the Fiske-Subbarow reagents to detect P_i produced when the gel is immersed in MgATP. In the modified method, the blue-colored zone of reduced phosphomolybdate is allowed to develop for about 8 min and the gel is then removed, rinsed with H_2O, and left in the open air. When this is done, the blue-colored zone corresponding to the position of the ATPase continues to intensify, while very little background color develops. As little as 0.01 unit of ATPase can be easily detected by this procedure.

Gel analyses under dissociating conditions were carried out in 10 or 12% polyacrylamide containing 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.1 M phosphate, pH 7, essentially according to Weber and Osborn (1969). The ATPase sample and standard proteins were heated at 100 °C for 3 min in 5% sodium dodecyl sulfate, 10% mercaptoethanol, and 0.01 M phosphate, pH 7.0.

Chymotrypsin Treatment. We used three times crystallized bovine pancreas α-chymotrypsin purchased from Sigma (St. Louis, Mo.). To interrupt chymotrypsin action we added

[1] Abbreviations used: CHY-ATPase refers to the fully active chymotrypsin-modified soluble ATPase; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane; PEI, polyethylenimine.

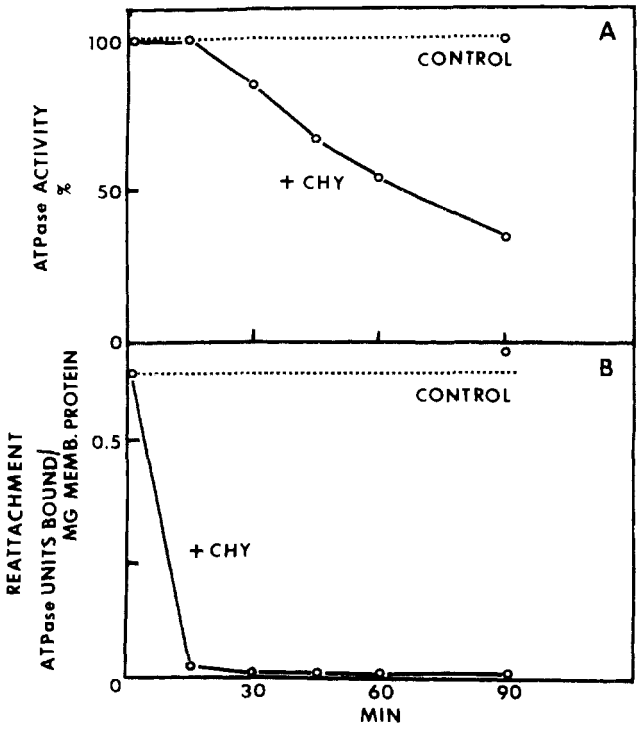

FIGURE 1: The time course of chymotrypsin action on ATPase activity and rebinding to membranes. ATPase (23 units) was incubated at 38 °C with 14 μg of chymotrypsin in 0.5 ml of 20 mM Tris-Cl buffer (pH 7.5) and 10 mM $MgCl_2$. Samples were taken for analysis at the indicated time intervals and phenylmethanesulfonyl fluoride was added to block further proteolysis. The effects on ATPase activity and reattachment to membranes are shown in the upper and lower panel, respectively. Analyses of control samples treated in the same way, except that chymotrypsin was omitted, are shown by the dotted lines. For the assay of reattachment, 0.3 unit of *active enzyme* remaining after chymotrypsin action was added to 0.25 mg of depleted membrane protein. The amount of bound enzyme was then determined by the pelleting assay as described previously (Abrams et al., 1974).

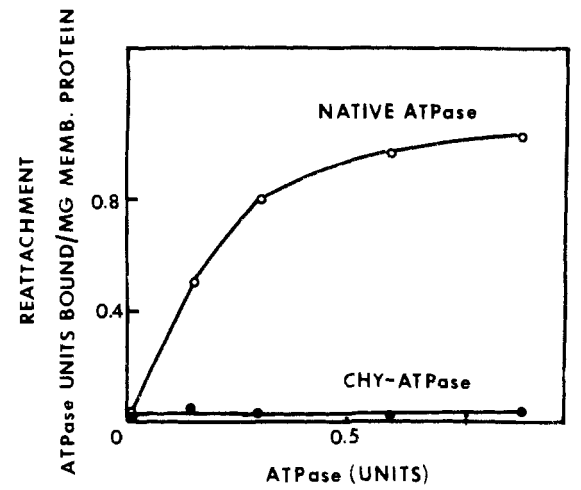

FIGURE 2: Membrane reattachment of native and chymotrypsin-modified ATPase as a function of enzyme concentration. Native and chymotrypsin-modified ATPase were purified by preparative gel electrophoresis in the presence of Mg^{2+} ions. Varying amounts of the native ATPase and CHY-ATPase were mixed with depleted membrane and the number of units bound was determined.

phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) at a level of about 0.1 to 0.3 mg/ml. $PhCH_2SO_2F$ was shown to prevent completely the chymotryptic conversion of the ATPase to the modified ATPase. It had no effect on the electrophoretic properties of the ATPase.

Reattachment Assay. The procedure for assaying rebinding of ATPase to depleted membranes has been described previously (Abrams and Baron, 1968; Baron and Abrams, 1971; Abrams et al., 1974). In essence, 0.3 unit is mixed with a suspension of depleted membranes (0.25 mg of protein) in 100 mM Tris-Cl (pH 7.5)–10 mM $MgCl_2$ in a total volume of 0.3 ml. The mixture is incubated for 30 min at 38 °C and centrifuged. The pellet is washed with the Tris-Mg^{2+} buffer, resuspended, and then assayed for bound ATPase activity.

Results

Time Course of Chymotrypsin Action on ATPase Catalytic Activity and Rebinding to Depleted Membranes. Figure 1 shows the time course of chymotrypsin action on both the ATPase catalytic activity (Figure 1A) and the capacity of the enzyme to rebind to depleted membranes (Figure 1B). With regard to the effect on ATPase activity, the results reveal that there was a 15-min period during which no significant change occurred. After this lag phase there was a gradual diminution of catalytic activity over a period of about 2 h. By contrast the rebinding capacity of the ATPase disappeared completely during the first 15 min of chymotrypsin action. Thus chymotrypsin appeared to remove a part of the protein needed for membrane binding without affecting the catalytic site.

The binding defect in the chymotrypsin-modified ATPase seems not to be due simply to a lowered binding efficiency. This is shown by measurements of binding as a function of enzyme concentration (Figure 2). With the native ATPase a typical saturation type binding curve was obtained (Abrams and Baron, 1968). On the other hand the chymotrypsin-modified ATPase did not bind significantly even at the highest levels of enzyme tested. This result points to the complete elimination by chymotrypsin of a structural feature in the ATPase that is essential for binding. As discussed in a later section this structural feature seems to be a small terminal segment of the three α chains.

Electrophoretic Characterization of the Chymotrypsin-Modified ATPase (CHY-ATPase). Figure 3 illustrates some typical electrophoretic analyses of the ATPase in regular gels before and after incubation of the enzyme with chymotrypsin. The conditions chosen for chymotrypsin treatment did not cause significant loss of catalytic activity. Staining of the gels for protein (panels A and B) and for ATPase activity (panels C and D) shows that the enzyme was converted to a catalytically active form that migrates about 15% faster than the native ATPase. As can be seen in panels A, B, and C of Figure 3, only a single new protein is formed and that it is catalytically active. We have designated this chymotrypsin-modified active ATPase as CHY-ATPase. It can also be seen in panels A, B, and C that the conversion to CHY-ATPase was virtually quantitative as no protein or catalytic activity remained in the position corresponding to that of the native ATPase.

Figure 3 (panel D) illustrates an electrophoretic analysis of the chymotrypsin-treated ATPase that had not undergone complete conversion to CHY-ATPase. Staining for ATPase activity reveals the presence of intermediate active bands that fall between the position of the native enzyme and the CHY-ATPase. The probable nature of these transient forms of the enzyme will be discussed later.

ATPase samples taken at different times during incubation with chymotrypsin (see Figure 1) were electrophoresed on gel slabs which were then stained for ATPase activity (Figure 4). At the shortest time of chymotrypsin action (15 min), the original enzyme band was no longer detectable in the gel and

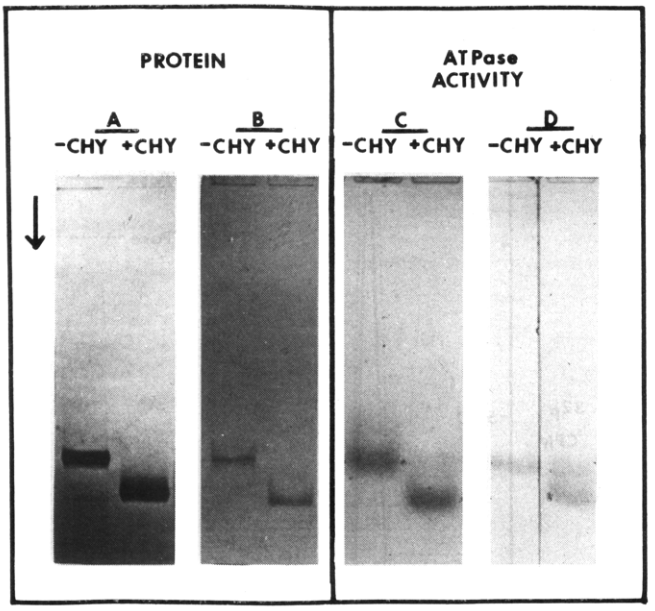

FIGURE 3: Effect of chymotrypsin on the electrophoretic profile of *S. faecalis* ATPase. Various native ATPase preparations and the corresponding chymotrypsin-modified enzyme were run on regular 5% polyacrylamide gels at pH 8 and stained for protein and/or ATPase activity as indicated. (A) Native ATPase (37 units/ml) was incubated with 5 μg/ml chymotrypsin for 30 min. (B) Native ATPase (4 units/ml) was incubated with 4 μg/ml chymotrypsin for 60 min. (C) Same gel as B showing ATPase activity prior to staining for protein (Abrams et al., 1975). (D) Native ATPase (35 units/ml) was incubated with 4 μg/ml chymotrypsin for 60 min; note the intermediate active forms.

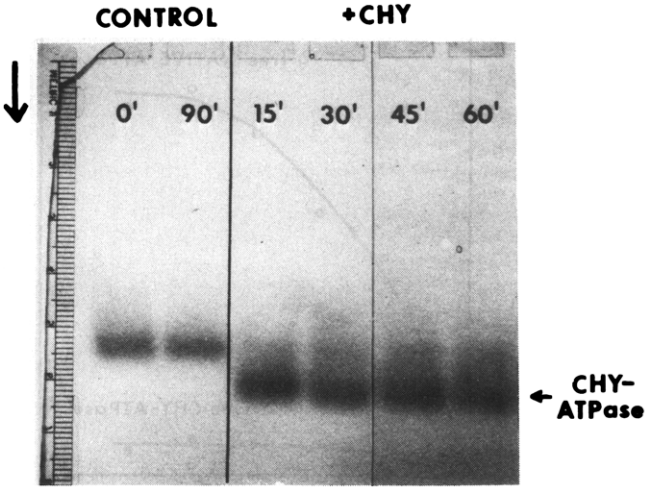

FIGURE 4: Gel electrophoretic analysis of ATPase after different times of chymotrypsin action. Native ATPase (45 units/ml) was incubated with chymotrypsin (26 μg/ml) at 38 °C in 20 mM Tris-Cl–10 mM $MgCl_2$, pH 7.5. At the indicated times samples of the native and chymotrypsin-treated enzyme were run on a regular slab gel in Tris-glycine, pH 8, and 2 mM $MgCl_2$. The gel was stained for ATPase activity (Abrams and Baron, 1967).

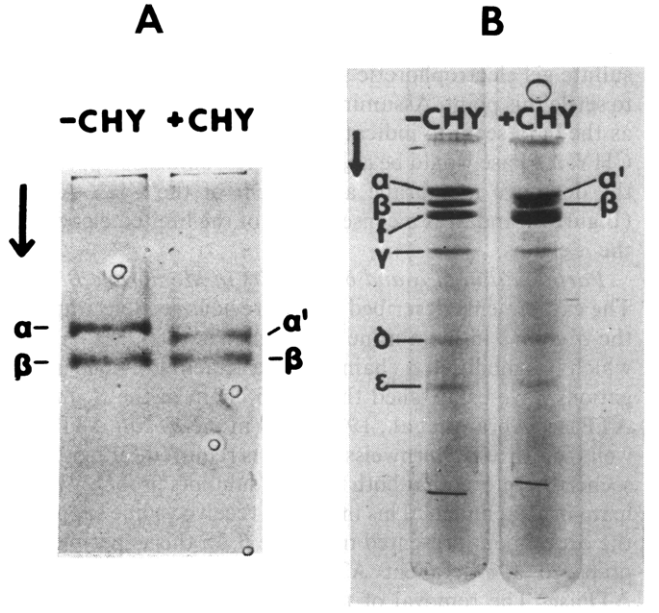

FIGURE 5: (A) Modified α chains in CHY-ATPase. Native and CHY-ATPase were analyzed in a 10% polyacrylamide slab gel under dissociating conditions. Because a small amount of enzyme was analyzed only the major subunits can be seen. From a comparison with standard proteins run on the same slab, the α, α', and β chains had molecular weights of about 55 000, 53 000, and 50 000, respectively. (B) Subunit analysis of native and CHY-ATPase. Native and CHY-ATPase preparations, purified by preparative gel electrophoresis, were analyzed on a 12% polyacrylamide cylindrical gel under dissociating conditions. The change in α to α' can be seen but there is no detectable change in the other subunits, β, γ, δ, and ϵ. (The 49 000-dalton protein, designated f, is derived from an inactive protein contaminant believed to be formed by proteolytic nicking of the major ATPase subunits in the cell or during the purification procedures (Abrams et al., 1975).)

was replaced totally by a single faster moving active form, CHY-ATPase. After the formation of CHY-ATPase, longer incubation with chymotrypsin caused only a gradual diminution of the CHY-ATPase band and no other active bands appeared. Evidently the CHY-ATPase accumulates as virtually the only product because it is relatively resistant to further chymotryptic action. Clearly the 15-min period during which no change in catalytic activity occurred (Figure 1) corresponds to the conversion of the ATPase to the faster moving active form, CHY-ATPase, visualized by the electrophoretic analysis (Figure 4). As will be seen in the next section, the formation of CHY-ATPase is correlated with a limited cleavage of the three α chains.

Subunit Structure of CHY-ATPase. As described in the previous sections, chymotrypsin action converts the ATPase to a new electrophoretic form, CHY-ATPase, which is fully active but defective in membrane attachment. It was of interest to correlate these changes with changes in the primary structure of the subunits. Figure 5A illustrates a gel electrophoretic analysis of the native ATPase and the CHY-ATPase, under dissociating conditions in a slab gel. In this analysis only small amounts of the enzyme were analyzed so that only the major subunits, α and β, are visualized. It is evident that all the α chains (ca. 55 000 daltons) disappeared completely and were replaced by a single type of modified α chain about 2000 daltons smaller. We have designated the chymotrypsin-modified α chain as α'. It is evident also from Figure 5A that chymotrypsin caused no discernible change in the β chains.

Figure 5B shows the subunit analysis of another ATPase preparation and its corresponding CHY-ATPase derivative in a cylindrical gel. In this analysis the minor subunits, γ, δ, and ϵ, as well as the major subunits, α and β, could be detected. Although the α and β chains are somewhat overloaded on the gel, one can see again the conversion of α to α'. It is also evident that all the minor subunits are still present in the CHY-ATPase with no discernible alteration.

It would appear that in CHY-ATPase only the α chains are cleaved, but it cannot be excluded that there may be cleavages in the other subunits too small to be detected by sodium dodecyl

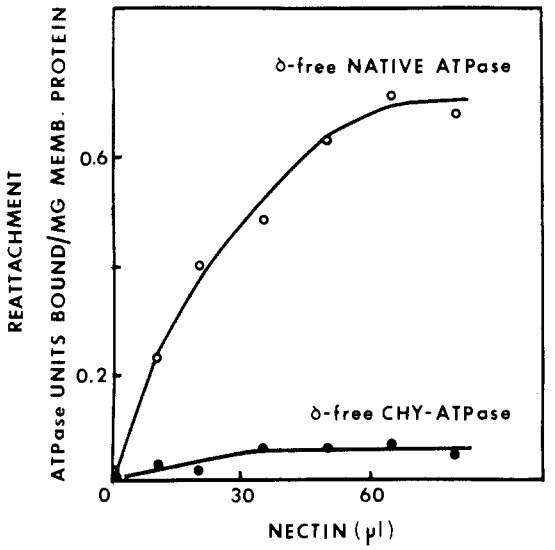

FIGURE 6: Effect of added nectin on rebinding of δ-free native ATPase and δ-free CHY-ATPase to membranes. Native ATPase and CHY-ATPase were freed of the δ subunit by preparative gel electrophoresis in the absence of Mg^{2+} ions (Abrams et al., 1976). The δ-free native ATPase and the δ-free *CHY-ATPase* were then assayed for membrane rebinding capacity in the presence of varying amounts of nectin (Baron and Abrams, 1971).

sulfate gel electrophoretic analysis. Further work is required to settle this point. Assuming that α is the only altered chain, as the data seem to indicate, the subunit composition of the CHY-ATPase would be $\alpha'_3\beta_3\ \gamma\ \delta\ \epsilon$. It would also appear that the defective membrane attachment of the CHY-ATPase (Figures 1 and 2) is a consequence of the limited cleavage of the α chains.

Participation of α and δ Subunits in Membrane Binding. The experiments described in the previous sections implicate the α chains in membrane binding since the CHY-ATPase which has modified α chains fails to attach. Previous investigations have established that the δ chain in the *S. faecalis* ATPase (Abrams et al., 1976), and in the *E. coli* ATPase as well (Smith and Sternweiss, 1975), is required for binding. It seems, therefore, that both types of subunits probably participate in attachment. This inference received some support by the experiment presented in Figure 6. In this experiment we prepared a δ-deficient ATPase and a δ-deficient CHY-ATPase. The removal of the δ chain was accomplished by preparative gel electrophoresis of the native ATPase and CHY-ATPase using gels that did not contain Mg^{2+} ions (Abrams et al., 1976). The δ-deficient ATPase and the δ-deficient CHY-ATPase were then compared for their ability to bind to depleted membranes when the δ subunit is added back. The δ subunit was added in the form of nectin[2] which was isolated from native ATPase according to Baron and Abrams (1971). The results (Figure 6) showed that the δ-deficient native ATPase rebinds to membranes as expected when nectin is added. However, the δ-deficient CHY-ATPase, which has modified α chains, did not rebind when nectin was added. If it is assumed that nectin can reassociate with the modified ATPase as it does with the native enzyme, then the results support the view that binding of the enzyme to the membrane depends on both δ and α chains.

[2] Sodium dodecyl sulfate gel electrophoresis of nectin preparation shows it is made up of δ chains and that it is probably a δ dimer (unpublished experiments and Abrams et al., 1976).

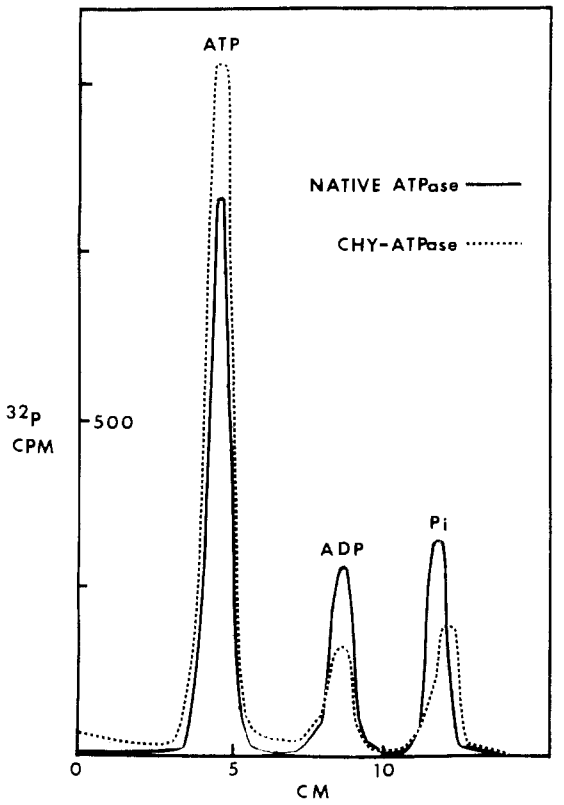

FIGURE 7: Tightly bound nonexchangeable nucleotide in native and CHY-ATPase. ^{32}P-labeled CHY-ATPase was prepared from ^{32}P-labeled native ATPase as described in the text. The bound ^{32}P ligands in the native and CHY-ATPase were released from the protein by acid denaturation in 0.01 N HCl and analyzed by thin-layer chromatography on polyethylenimine sheets prewashed with methanol (Randerath and Randerath, 1967). Unlabeled markers, ATP, ADP, AMP, and P_i were added to the samples. After application of the samples, the PEI sheets were dipped in methanol in order to remove salts which might otherwise interfere with the analysis. Chromatography was done in 0.75 M phosphate buffer, pH 3.4 (Sy, 1974). The position of the marker nucleotides was located by UV light and the radioactive profile was determined by liquid scintillation counting of 0.5-cm scrapings in a standard toluene-based scintillation system.

Retention of Tightly Bound Nonexchangeable Nucleotide in the CHY-ATPase. We were interested in determining if tightly bound nonexchangeable nucleotide present in the native *S. faecalis* ATPase (Abrams et al., 1975) is retained when the enzyme is converted to CHY-ATPase. ^{32}P-labeled ATPase was isolated from cells that had been incubated in [^{32}P]orthophosphate (Abrams et al., 1973) and then purified to near homogeneity by DEAE-cellulose chromatography. A portion of the ^{32}P-labeled ATPase was converted to [^{32}P]CHY-ATPase. The conversion to CHY-ATPase was verified by gel electrophoresis in a regular gel which showed complete conversion to the faster moving active form. The ^{32}P-labeled native and CHY-ATPase were dialyzed against pH 7.5 Tris buffer containing unlabeled ATP, ADP, and P_i, and the labeled ligands in the native and the CHY-ATPase were then determined. The results (Figure 7) show that the CHY-ATPase like the native enzyme contained [^{32}P]ATP, [^{32}P]ADP, and $^{32}P_i$ with the ATP being by far the predominant component. These ligands are referred to as nonexchangeable because they are not displaced from the enzyme by a mixture of nonradioactive ATP, ADP, and P_i, each at 0.1 mM, added at several steps during the purification of the enzyme (Abrams et al., 1975). Since the content of nonexchangeable nucleotides (and P_i) is unchanged when the native ATPase is converted to CHY-

ATPase, it is obvious that these ligands are not located in the segment of the α chains split off by chymotrypsin. It is also evident that the limited proteolytic cleavage of the α chains does not perturb the structure of the enzyme sufficiently to cause release of the tightly bound nucleotide ligands or to cause them to become exchangeable.

Discussion

Through the use of chymotrypsin, we have obtained evidence that a small segment of the α chains in the *S. faecalis* ATPase is needed for attachment of the enzyme to the plasma membrane. A notable feature of the action of chymotrypsin on the enzyme is that the initial proteolytic attack is remarkably limited and apparently confined to the three α chains (Figures 3, 5A, and 5B). The modified enzyme, designated CHY-ATPase, is formed in essentially quantitative yield and remains fully active. It is easily recognized by gel electrophoresis as a single protein band which exhibits ATPase activity and which migrates somewhat faster than the native ATPase (Figure 3). During the very earliest stages of chymotrypsin action, before the conversion to CHY-ATPase is complete, some transient active ATPase forms with intermediate electrophoretic mobilities can be observed. Prolonged incubation with chymotrypsin causes only a slow degradation of the CHY-ATPase to inactive products. Since the CHY-ATPase is formed relatively rapidly and is fairly resistant to further chymotryptic proteolysis, it tends to accumulate as virtually the only product.

In the conversion of native ATPase to CHY-ATPase each of the three 55 000-dalton α chains is cleaved to give a modified chain, designated α', about 2000 daltons smaller (Figure 5A). From this observation and the foregoing considerations, we may formulate the action of chymotrypsin on the ATPase substructure as shown in Scheme I.

SCHEME I.

$$\text{native ATPase} \xrightarrow{\text{fast}} \text{intermediates} \xrightarrow{\text{fast}} \text{CHY-ATPase}$$

$$\xrightarrow{\text{slow}} \text{inactive products}$$

$$(\alpha_3\beta_3\gamma\delta\epsilon) \qquad \left\{ \begin{array}{c} \alpha'_1\alpha_2\beta_3\gamma\delta\epsilon \\ \alpha'_2\alpha_1\beta_3\gamma\delta\epsilon \\ \hline \text{etc.} \end{array} \right\} \qquad (\alpha'_3\beta_3\gamma\delta\epsilon)$$

As indicated in the scheme, the transient active intermediates probably consist of a mixture of species in which the α chains are in various stages of conversion to α'. The 2000-dalton segment of the α chains removed by chymotrypsin is clearly far more susceptible to attack than is the remainder of the α chain. It seems likely then that the chymotrypsin-sensitive segments in the three α chains protrude from the enzyme surface as exposed "tails".

There is no evidence that any of the other subunits besides the α chains are altered in the CHY-ATPase (Figure 5B). Moreover, there seems to be no gross conformational distortion in the CHY-ATPase since it retains full catalytic activity and also the full complement of tightly bound nucleotide present in the native ATPase (Figures 1 and 7). On the other hand, the CHY-ATPase lacks completely the ability to reattach to membranes (Figure 1). These findings imply that the chymotrypsin-sensitive "tails" in the three α chains contain amino acid residues which interact with a corresponding set of specific receptor sites in the membrane. In previous work, the δ chain in the *S. faecalis* ATPase was implicated in membrane attachment (Abrams et al., 1976). It is reasonable to suggest that the three α chains and the δ chain act in concert to provide multiple attachment points in the enzyme and thereby bring about a firm association with the membrane. Using a single ATPase preparation some additional evidence for the involvement of both δ and α chains in membrane binding was obtained. It was found that a δ-deficient ATPase preparation rebinds to membranes normally if the isolated δ chain (in the form of nectin) is added. However, a δ-deficient *CHY-ATPase* preparation did not rebind at all when nectin was added, presumably because the α chains had been cleaved.

This is the first report that relates a portion of the ATPase primary structure to a functional property of the enzyme, namely, membrane binding. As little is known about the nature of the binding interaction, the amino acid composition and sequence of the chymotrypsin-sensitive peptide segment of the α chain, consisting of about 20 amino acid residues, should prove to be of great interest.

Acknowledgments

We acknowledge the technical assistance of Mr. Robert Wolfson and Mr. Scott Wheeldon.

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A Proposal for the Mechanism of Chymotrypsinogen Activation†

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ABSTRACT: Carboxymethylation of Met-192 of chymotrypsin with iodoacetate inhibits the hydrolysis of acetyltyrosine-*p*-nitroanilide and of acetyltyrosine ethyl ester but has no effect on the hydrolysis of carbobenzyloxytyrosine *p*-nitrophenyl ester or on the reaction with active site titrants. In chymotrypsinogen, Met-192 is largely unreactive toward iodoacetate, whereas Met-180 reacts slowly. Prior acylation of Ser-195 has little effect on the rates of subsequent alkylation of the zymogen or the enzyme. These results, taken together with previous kinetic, spectral, and crystallographic data, lead to a model of zymogen activation which entails not only an enhancement of substrate binding but also a mechanism for stabilization of the transition state during catalysis. In this model, the absence of the "oxyanion hole" in the zymogen precludes effective catalysis even when the substrate is bound.

It has been recently demonstrated that, contrary to traditional views, certain zymogens are not enzymatically inert but possess an intrinsic activity which upon activation becomes magnified by several orders of magnitude (Morgan et al., 1972; Kassell and Kay, 1973; Gertler et al., 1974a). Chymotrypsinogen and trypsinogen react stoichiometrically with active site titrants, such as Dip-F[1] or CH_3SO_3F, and the hydrolysis of the pseudosubstrate *p*-nitrophenyl-*p'*-guanidinobenzoate (NPGB) by chymotrypsinogen proceeds via the same type of acyl intermediates as does the chymotrypsin-catalyzed reaction. Kinetic analyses, including competitive inhibition studies, as well as spectral probes have lead to the conclusion that the principal impairment of the catalytic effectiveness of these zymogens, as compared with the corresponding enzymes, is a diminished binding of the substrate rather than a deficiency in the catalytic elements per se (Gertler et al., 1974a,b; Kerr et al., 1975). This view is in accord with the interpretations of the differences between the electron density maps derived from x-ray structure analysis of chymotrypsinogen and α-chymotrypsin (Freer et al., 1970; Matthews et al., 1967; Steitz et al., 1969). The most significant difference appears to be a distortion in the zymogen of the substrate binding pocket (also referred to as tosyl hole) which in the enzyme accommodates the side chain of the amino acid which contributes the carbonyl group to the susceptible peptide bond. The formation of the specificity pocket results from the repositioning of Met-192 from a deeply buried position in the zymogen to the surface in the enzyme, but the distortion of this pocket alone may not be sufficient to explain the enormous difference in catalytic activity between zymogen and enzyme (Wright, 1973a,b).

We have recently reported that the intrinsic enzymatic activity of chymotrypsinogen toward NPGB was increased approximately sevenfold upon oxidation of Met-192, due to increased affinity of the enzyme for the substrate (Gertler et al., 1974b). While this change is minute, when compared with that accompanying the normal zymogen activation, it is at least in the same direction. Attempts to extend these studies to another type of modification of Met-192, i.e., by carboxymethylation (Koshland et al., 1962), lead us to the conclusion that the rate of this reaction is a sensitive probe of the topography of the region of the substrate binding site and of its involvement in the catalysis by chymotrypsinogen.

Materials and Methods

α-Chymotrypsin and chymotrypsinogen A were obtained from Worthington Biochemicals. [1-^{14}C]Iodoacetic acid was a product of New England Nuclear Corp. Substrates and active-site titrants were obtained as described previously (Kerr et al., 1975).

Carboxymethylation of chymotrypsin, chymotrypsinogen, and their derivatives was carried out by incubation of the proteins (54 μM) at 37 °C with radioactive iodoacetate (54 mM) in 0.2 M acetate buffer, pH 4.5. Incorporation of radioactivity into the proteins was measured, after desalting over Sephadex G-25 in 1 mM HCl, using a Packard 574 liquid scintillation counter. Protein concentrations were calculated from the absorbance at 280 nm or by the method of Lowry et al. (1951).

Amino acid analysis of the radioactive products of acid hydrolysis of the proteins was carried out on a Beckman 120B

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[1] Abbreviations used: NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; pGB, *p*-guanidinobenzoyl; NPSA, *p*-nitrophenyl-*p'*-(dimethylsulfonioacetamido)benzoate; pSA, *p*-(dimethylsulfonioacetamido)benzoyl; MUTMAC, methylumbelliferyl-*p'*-trimethylammonium cinnamate; pTMAC, *p'*-trimethylammoniumcinnamoyl; CD, circular dichroism; Dip-F, diisopropyl fluorophosphate; CH_3SO_3F, methylsulfonyl fluoride.