EFFECTS OF TRYPSIN TREATMENT ON THE STRUCTURE AND FUNCTION OF SOLUBILIZED COUPLING FACTOR-LATENT ATPASE FROM *MYCOBACTERIUM PHLEI*

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SUMMARY: The effect of trypsin treatment on the solubilized coupling factor-latent ATPase from *Mycobacterium phlei* was studied. Maximal stimulation of ATPase activity by trypsin is accompanied by a decrease of about 20,000 daltons in molecular weight and a complete loss of the ability to rebind to depleted membranes. There is also conversion of the A subunit of the latent enzyme to an A'' form via an A' intermediate. The increase in ATPase activity, loss of coupling factor activity, and loss of rebinding capacity changed in a different manner in response to partial degrees of trypsin activation, indicating that each of these functions may have a different structural requirement.

INTRODUCTION: The coupling factor-latent ATPase (BCF_4) from *Mycobacterium phlei* (analogous to mitochondrial F_1) exhibits a dramatic enhancement of ATPase activity and a complete loss of coupling factor activity following solubilization from membrane vesicles (1) and trypsin treatment (2,3). Following trypsin treatment, a 35% reduction in ADP binding was observed (5). Trypsin similarly elicits the ATPase activity of membrane-bound BCF_4, but without loss of coupling factor activity (3). Since the structural basis for the loss of ADP binding by soluble BCF_4 and the alterations which lead to the differential effect of trypsin on ATPase and coupling factor activities are unknown, it was important to examine structural alterations that accompany these observed changes in function. It was also important to determine whether such structural alterations could account for these observed functional changes.

It was equally important to ascertain whether trypsin-treated BCF_4 could rebind to membrane vesicles (DETP) depleted of the enzyme and whether the observed

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loss of coupling factor activity could be accounted for by a loss of ability to rebind to membranes. In addition, other factors, such as reduced ADP binding, may contribute to the loss of coupling factor activity. In studies dealing with such factors, it would be essential to examine their effect on coupling factor activity under conditions where loss of rebinding was not significant.

MATERIALS AND METHODS: Growth conditions and preparation of electron transport particles (ETP) from *M. phlei* (ATCC 354) have been previously described (6). Coupling factor-latent ATPase was solubilized by suspending ETP in 0.25 M sucrose in the absence of metal ions, followed by centrifugation at 105,000 x g for 90 min. The resulting pellet is the depleted membrane vesicles (DETP); the sucrose supernatant fraction exhibits coupling factor activity when reconstituted with DETP and is referred to as BCF_4 (1).

Activation ("unmasking") of the latent ATPase activity of BCF_4 by trypsin treatment and assay of latent ATPase activity was carried out as described earlier (2). Partial activation was achieved by reducing the amount of trypsin and/or time of treatment at 30°C, or by exposing the sample to an immobilized preparation of trypsin (Enzite-EMA-Trypsin, a copolymer of trypsin and ethylene malic anhydride) for intervals of time ranging from one to 20 minutes.

Measurement of coupling factor activity (1) and sodium dodecyl sulfate (SDS) gel electrophoresis procedures (4) were performed as described previously. The following proteins were used as molecular weight standards to calibrate 10% acrylamide SDS gels: bovine serum albumin (68,000), bovine liver catalase (57,500), ovalbumin (43,000), yeast alcohol dehydrogenase (37,000), soybean trypsin inhibitor (23,000), and pancreat trypsin (15,000). Protein concentration was estimated by the method of Lowry, et al. (7). Molecular weight estimates of latent and trypsin-activated BCF_4 were obtained by the pore gradient gel electrophoresis method as described (5). In the present study, retardation coefficient (K_R) data for the standard proteins was input to the FORTRAN I RADKRI program of Rodbard and Graber (8). The standard curve was a computer-generated regression of the square root of K_R upon the molecular radius ($\bar{R}$) and retardation coefficients of unknown proteins were referred to this curve to obtain estimates for their molecular radii and molecular weight.

Membrane rebinding ability of BCF_4 was measured by incubating control and trypsin treated samples with thoroughly-washed DETP at a ratio of 0.25 mg BCF_4 per mg DETP. The mixture was centrifuged at 105,000 x g for 90 min in the presence of 4 mM $MgCl_2$, and the percent of the total ATPase activity rebound to the DETP pellet and remaining unbound in the supernatant was determined.

RESULTS AND DISCUSSION: Molecular weight estimates from the computer-generated standard curve yielded a value of 401,000 for latent BCF_4 and 381,000 for the trypsin-activated enzyme. Error estimates for this method of molecular weight analysis are comparable to error estimates by standard methods, i.e., about 10% (8). The observed difference in molecular weight between latent and activated BCF_4 (about 20,000) was nevertheless distinct and reproducible.

The subunit composition of latent BCF_4, determined from carefully calibrated

7.5% and 10% SDS gels, was: A (64,000), B (53,000), C (33,000), D (14,000), and E (8,000). These figures differ from earlier estimates (4), and are considered to be more accurate. The SDS gel pattern of the trypsin-activated enzyme was similar to that for latent BCF_4 except for replacement of the A subunit by an A'' (58,000) species. Although the 10,000 - 20,000 molecular weight region of gels for trypsin-treated samples was obscured by the presence of trypsin and trypsin inhibitor, gels of samples activated by immobilized trypsin clearly showed the presence of the D (14,000) subunit. There was an additional intermediate species A' (61,000) present in partially activated samples, and the A, A', and A'' species were present in different samples in proportions that reflected the degree of activation.

Subunit stoichiometry was estimated from the relative areas of subunit peaks on densitometer scans of Coomassie blue-stained gels. A reproducible ratio of 3:3:1 was seen for A:B:C (latent enzyme); similarly, a 3:3:1 ratio was seen for A'':B:C for the trypsin-activated enzyme. Stoichiometric analysis based on protein staining is subject to error due to possible differential dye binding (11). Sample gels of latent and trypsin-treated BCF_4 were therefore stained with Fast Green, Procion Blue, and Amido Black; the ratios of both A:B and A'':B were verified as close to 1:1 for these stains also.

Based on this data, the subunit composition for the latent BCF_4 enzyme is proposed to be A_3B_3CDE, which becomes A''_3B_3CDE after trypsin activation. The calculated molecular weight of the latent enzyme based on subunit molecular weight and the proposed composition was 406,000, and the calculated value for the activated enzyme was 388,000. A loss of 6,000 from each A subunit would give a net loss of 18,000 per mole for the fully activated enzyme. The calculated molecular weight for latent BCF_4 was thus in excellent agreement with the experimental molecular weight (401,000) and with the experimental value obtained earlier for an affinity column-purified preparation (404,000 $\pm$ 6%) (5). The calculated molecular weight for the trypsin-activated enzyme was likewise in agreement with the experimental estimate (381,000), and calculated and experimental values for

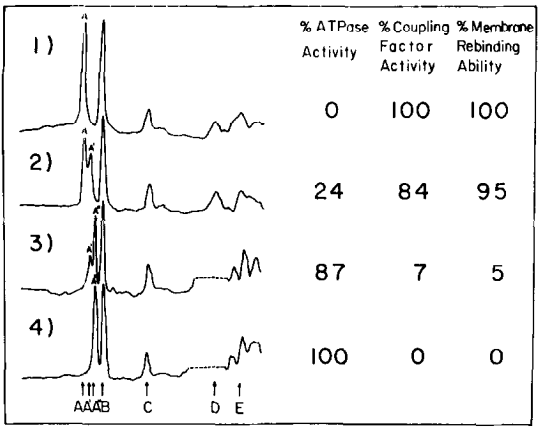

Figure 1. Subunit structure and functional properties of solubilized *M. phlei* BCF_4 following trypsin treatment.

Densitometer scans of Coomassie blue-stained SDS gels, percent ATPase activity, percent coupling factor activity, and percent membrane rebinding ability are given for: 1) untreated (latent) BCF_4; 2) BCF_4 treated with 2.4 mg Enzite-EMA-Trypsin per mg BCF_4 for 20 min at 30°C; 3) BCF_4 treated with 0.17 mg pancreatic trypsin per mg (protein:trypsin=6:1) for 2 min at 30°C; and 4) BCF_4 treated with 0.17 mg trypsin per mg for 6 min at 30°C.

the molecular weight difference were similar (18,000 vs. 20,000).

Under conditions where latent BCF_4 was shown to rebind fully to DETP membranes (see METHODS), the trypsin-treated activated enzyme did not rebind (Fig. 1). Changes in structure and function of BCF_4 produced by trypsin treatment were examined more closely by assaying the same sample of partially activated BCF_4 for ATPase activity, coupling factor activity, rebinding ability, and SDS gel pattern. Fig. 1 gives representative gel scans and functional values (expressed as % maximum) for 1) latent (untreated) BCF_4; 2) BCF_4 after 24% activation; 3) BCF_4 after 87% activation; and 4) fully activated BCF_4.

The gel pattern for 24% activated BCF_4 (Fig. 1) is similar to the pattern reported earlier (4) for a BCF_4 sample assumed to be latent and intact. However, when a fresh sample of BCF_4 in 0.25 M sucrose (i.e., with subunit A in the A form only) was stored at 4°C for 14 days in 4mM $MgCl_2$, a 40% conversion of A to A' was seen. The A' species did not form under the same conditions in the absence of $MgCl_2$

and presence of 2 mM phenyl methyl sulfonyl fluoride and 0.05% ethanol. These results indicated that the species previously identified as a "B" subunit was actually A' formed by endogenous or maverik protease activity. Similar observations were made by Files and Weber for the Escherichia coli lac repressor protein (10).

It is apparent that each function in Fig. 1 changed in a different way in response to trypsin treatment and that loss of coupling factor activity did not parallel the loss of rebinding ability during initial stages of activation. At an early stage of activation (24%), a larger gain in ATPase activity relative to the loss of coupling factor activity (16% decrease) is apparent. The loss of rebinding at this stage was minimal (5%). The major structural change observed at this stage was the conversion of the A subunit to A'. This change in structure thus appeared to be accompanied by a conformational change in which the molecule possessed both ATPase and coupling factor activities (partially expressed) as well as an ability to rebind to membranes.

At a later stage of activation (87%), there was a large decrease in both coupling factor activity and rebinding. The concomitant major structural change was conversion of A' to the stable A" specie. Since coupling factor activity is dependent upon the ability of the molecule to rebind, the only conclusion that may be drawn from this data is that the observed structural change in the A subunit induces a molecular conformational state in which the enzyme cannot rebind to the membrane. The ability to synthesize ATP may have also been altered, but such a change could not be distinguished from the effect on rebinding in the present study. Since the delta subunit of other energy-transducing ATPases has been shown to mediate attachment of the enzyme to the membrane (11), the "D" subunit of BCF_4 may have a similar role. If so, it is possible that the conformational changes responsible for loss of rebinding may also include an alteration of the D subunit which is not apparent.

A recent study by Leimgruber and Senior (12) on soluble mitochondrial coupling factor (F_1) showed that mild trypsin treatment produced a loss of coupling factor activity correlated with a loss of tightly-bound ADP under conditions where membrane

rebinding was normal. Although major changes in subunit structure were not observed, it is possible that an altered form of subunit #1 was generated under these condition comparable to the A' species seen for trypsin-treated BCF_4. Formation of a stable A' product from the A subunit of Streptococcus faecalis by chymotryptic digestion has also been reported (13); the conversion was accompanied by loss of membrane rebinding capacity with full retention of the (non-latent) ATPase activity and tightly bound nucleotides.

The in vitro trypsin activation of latent BCF_4 ATPase activity appeared to occur in a manner similar to the process by which zymogens are activated in vivo (14). If so, this would be the first demonstration of trypsin activation of an energy-transducing ATPase by this kind of mechanism. BCF_4 is also interesting because it has several functions that each responds in a different way to limited proteolysis. Certain bifunctional enzymes as well as the multifunctional ATPase of S. faecalis also exhibited differential functional effects in response to limited proteolysis (10,13,15). In each of these cases, there was specific and limited cleavage producing selective inactivation of certain function(s) and not other(s). The response of M. phlei BCF_4 to in vitro trypsin activation represents, on the other hand, a proenzyme-type of selective activation of one function with concomitant inactivation of other functions.

Since in vivo activation of latent ATPase must occur by a reversible process, these results may indicate only that a rearrangement of active site group(s) is necessary for this activation. This rearrangement may be induced indirectly by conformational changes in the molecule as a whole like those mediating the activation of ATPase by trypsin in vitro and activation of zymogens in vivo.

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