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TRYPSIN CLEAVAGE OF THE α-SUBUNIT OF BEEF HEART F_1-ATPase ABOLISHES ATP SYNTHESIS AND ATP-DRIVEN ENERGY-TRANSDUCTION CAPABILITIES

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Previous work has shown that mild trypsin treatment eliminates energy-transduction capability and tight (non-exchangeable) nucleotide binding in beef heart mitochondrial F_1-ATPase (Leimgruber, R.M. and Senior, A.E. (1976) J. Biol. Chem. 251, 7103–7109). The structural change brought about by trypsin was, however, too subtle to be identified by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis, and was not defined. In this work we have applied two-dimensional electrophoresis (isoelectric focussing then sodium dodecyl sulfate polyacrylamide gradient electrophoresis) to the problem, and have determined that the α-subunit of F_1 is altered by the mild trypsin treatment, whereas no change was detected in β-, γ-, δ- or ε-subunits. Binding of ADP to the trypsin-treated F_1 was compared to binding to control enzyme over a range of 0–40 μM ADP in a 30 min incubation period. There was no difference between the two enzymes, K_d^{ADP} in Mg^{2+}-containing buffer was about 2 μM in each. Since the tight (nonexchangeable) sites are abolished in trypsin-treated F_1, this shows that tight exchangeable ADP-binding sites are different from the tight nonexchangeable ADP-binding sites. There was no effect of trypsin cleavage of the α-subunit on β-subunit conformation as judged by aurovertin fluorescence studies. The cleavage of the α-subunit which occurred was judged to occur very close to the C- or N-terminus of the subunit and constitutes therefore a small and specific chemical modification which abolishes overall function in F_1 but leaves partial functions intact.

When pure soluble beef heart mitochondrial F_1-ATPase is treated with low amounts of trypsin for short time periods it loses the ability to catalyze ATP synthesis or ATP-driven energy transductions after reconstitution with F_1-depleted membranes [1,2]. Uncoupled ATPase activity in the soluble F_1, inhibition by ADP or the F_1-inhibitor protein, and binding of F_1 to F_1-depleted membranes are unimpaired. No change in mobility of any of the five subunits of F_1 was seen on single-dimensional SDS-polyacrylamide gels after trypsin treatment [2]. There did seem to be a correlation, however, between the loss of 'tightly bound' nucleotide from F_1, loss of energy-transduction capability and tryptic hydrolysis [2,3]. Therefore, it seemed likely that further examination of the trypsin effect would increase our understanding of the role of individual subunits in F_1. In this paper we describe the use of two-dimensional gel electrophoresis to identify the trypsin cleavage site.

Materials and Methods

Treatment of soluble beef heart F_1-ATPase followed the procedures described in Ref. 2. All experiments used 10 μg trypsin/mg F_1 and incubation was performed for 10 min at 20°C before stopping the reaction with a 5-fold excess of soybean trypsin inhibitor. F_1 treated in this way rebinds normally to ASU particles, regaining oligomycin sensitivity once

Abbreviations: ASU-particles, mitochondrial inner membrane vesicles depleted of F_1 by sequential exposure to NH_3, sonication, gel filtration and urea; SDS, sodium dodecyl sulfate.

bound (Ref. 1 and Leimgruber and Senior, unpublished data), but is totally depleted of tightly bound ADP or ATP [2] and cannot rebind either nucleotide [3]. Energy-transduction capability is lost completely [2,3]. Control F_1 samples were treated identically, except the soybean trypsin inhibitor was added before trypsin.

Two-dimensional gel electrophoresis utilizing isoelectric focussing in the first dimension and SDS-polyacrylamide gradient gel separation in the second dimension followed the procedure of O'Farrell [4]. After trypsin treatment and precipitation in $(NH_4)_2SO_4$, samples were dissolved in 9 M urea/2% (w/v) NP-40/5% (v/v) β-mercaptoethanol/2% ampholines (pH range 3.5–10). Samples were allowed to stand for 1 h at 20°C and were then applied to the isoelectric focussing gels. These gels were not prerun, the 20 mM NaOH was replaced by 5% (v/v) ethylenediamine, and the normal run time was 2 h at 1000 V (1.2 mA/tube). In the second dimension the acrylamide gradient was 10–23% (w/v) and the run time 80 mA · h.

Nonequilibrium pH gradient electrophoresis [5] was carried out by running the normal isoelectric focussing gels for 1.5 h at 500 V.

Labelling of F_1 with *N*-[^{3}H]ethylmaleimide was carried out as described by Senior [6].

Binding of [2,8-^{3}H]ADP to soluble F_1 was carried out using the centrifuge column procedure of Penefsky [7]. The trypsin-treated and control samples of F_1, after precipitation with $(NH_4)_2SO_4$ [2] were redissolved in 50 mM Tris-H_2SO_4, pH 8.0, and passed through a Sephadex G-25 column to remove free and loosely bound nucleotide [2]. Samples (100 μg) were then incubated in 300 μl of the same buffer containing [^{3}H]ADP, with either 2 mM $MgSO_4$ or 1 mM EDTA. After 30 min at room temperature, 100 μl were applied to the centrifuge columns and spun. The centrifuge columns were of Sephadex G-50 (fine) containing 50 mM Tris-H_2SO_4, pH 8.0, with either 2 mM $MgSO_4$ or 1 mM EDTA. Protein recovery was about 66%, and variable enough to necessitate analysis of each collected effluent. The volume, protein content and radioactivity of each effluent were measured. A molecular weight of F_1 of 360 000 was assumed.

Aurovertin D titration was performed as described by Chang and Penefsky [8].

Results

Structural studies. The two-dimensional gel system revealed that trypsin cleaves the α-subunit of F_1, causing it to increase slightly in p*I* as revealed by a shift in horizontal mobility and to decrease slightly in molecular weight as revealed by mobility in the vertical direction (Fig. 1). As seen in Fig. 1, the β-, γ-, and δ-subunits of F_1 were unchanged by trypsin treatment. The α- and γ-subunit were identified in this system from their mobility in the vertical direction, and from the fact that both were labelled by *N*-[^{3}H]ethylmaleimide in labelling studies. The β-subunit was localized by its mobility in the vertical direction. The ε-subunit is not seen in Fig. 1 because it has a very high p*I* [9]. The linear pH gradient in the gel in Fig. 1 ran from 4.2 to 9.6.

To study the ε-subunit, the nonequilibrium pH gradient electrophoresis approach [5] was used. The ε-subunit of trypsin-treated F_1 ran with exactly the same mobility as that the of the ε-subunit of control F_1 in the isoelectric focussing dimension of this system (data not shown). Since trypsin does not alter the mobility of the ε-subunit in one-dimensional SDS-polyacrylamide gels [2], we can conclude that the only subunit affected by trypsin is the α-subunit. The data suggest that cleavage of the α-subunit occurs close to either the C- or N-terminus.

ADP-binding studies. It is established that beef heart F_1 binds ADP reversibly with high affinity, especially in Mg^{2+}-containing buffer, when ADP is incubated with F_1 for periods of minutes. Over a period of hours, the ADP begins to become incorporated into the very tight, nonexchangeable sites [10]. Since trypsin-treated beef heart F_1 lacks the very tight nonexchangeable ADP-binding sites [2,3], it was of interest to measure reversible ADP binding over a period of 30 min. Fig. 2 shows the binding of ADP (0–40 μM) to both trypsin-treated and control F_1 in Mg^{2+}- or EDTA-containing buffer. These data are very similar to those of the binding curve published previously by Cantley and Hammes [11]. As is well established, binding of ADP in Mg^{2+}-containing buffer was much tighter than in EDTA. There was no significant difference between control and trypsin-treated F_1 in either buffer. Scatchard analyses of the data obtained from Mg^{2+}-containing buffer were linear and the data showed the control F_1 bound

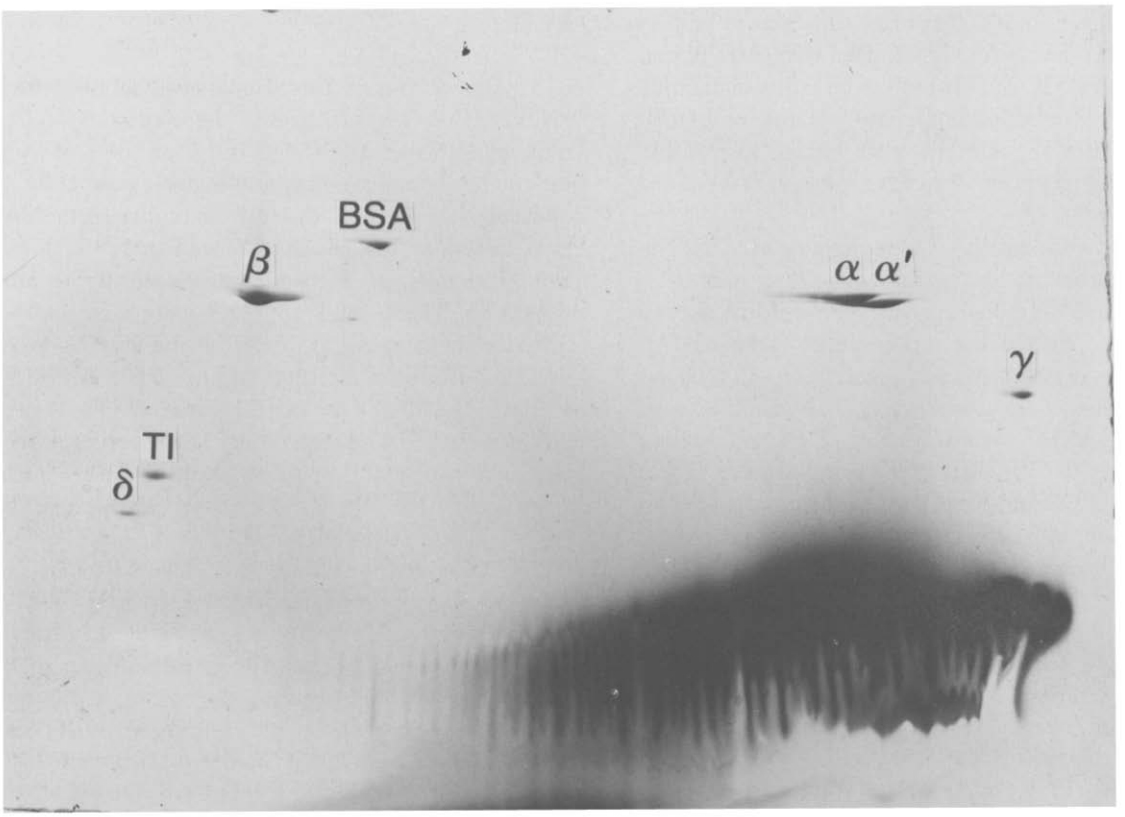

Fig. 1. Two-dimensional electrophoresis of trypsin-treated and control F_1. Isoelectric focussing in the horizontal direction, with alkaline end to the right; SDS-polyacrylamide gel electrophoresis in vertical direction. 5 μg of control F_1 were coelectrophoresed with 5 μg of trypsin-treated F_1, and 1 μg of bovine serum albumin was added as standard. Similar results were seen when 10 μg trypsin-treated and 10 μg control F_1 were coelectrophoresed. If trypsin-treated F_1 was run alone, only α′ was seen. If control F_1 was run alone, only α was seen. α–ε, corresponding subunits of F_1; α′, new α in trypsin F_1; BSA, bovine serum albumin; TI, trypsin-inhibitor protein.

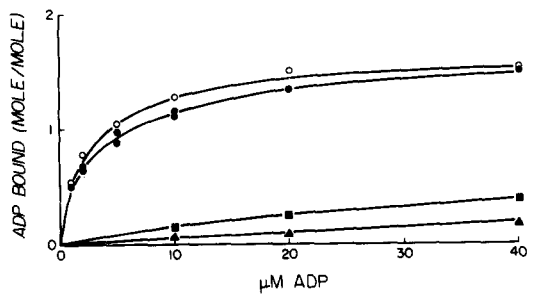

Fig. 2. Binding of [^{3}H]ADP to F_1. (○——○) Trypsin-treated and (●——●) control F_1 in the presence of Mg^{2+}; (▲——▲) control and trypsin-treated F_1 (same curve) in the presence of EDTA; (■——■) trypsin-treated and control F_1 (same curve) in the presence of EDTA with 10 μM aurovertin in incubation buffer and centrifuge column buffer. See Materials and Methods for procedures.

ADP with a K_d value of 2.15 μM, whereas the K_d with trypsin-treated F_1 was 2.0 μM.

Aurovertin-binding studies. In a recent study of *uncA* point mutants of *Escherichia coli*, the α-subunit of F_1 was found to be altered [12]. In a follow-up study [24] we have shown that the environment of the aurovertin-binding site is altered in *uncA* F_1 (the maximum fluorescence of the aurovertin-F_1 complex is about 70% increased) although the K_d and number of binding sites for aurovertin remain the same as those of normal (about 3 μM and 2, respectively). Aurovertin is known to bind to the β-subunit of both beef heart and *E. coli* F_1 [13,14].

We therefore studied aurovertin binding to control

and trypsin-treated beef heart F_1 samples, determining K_d, maximum fluorescence for the aurovertin-F_1 complex and n (number of aurovertin-binding sites) to find out if cleavage of the α-subunit had in any way changed the aurovertin-binding site. There was no difference between trypsin-treated and control F_1 in any of these parameters. Aurovertin had a small, reproducible effect in increasing ADP binding to F_1 in EDTA-containing buffer. This effect was present also in trypsin-treated F_1 (see Fig. 2).

Discussion

The data presented suggest that a small structural alteration in the α-subunit of beef heart F_1 produced by trypsin cleavage causes loss of ATP synthesis, ATP-driven energy transduction and tight (nonexchangeable) nucleotide-binding capabilities. The β-subunit was apparently unaffected structurally by trypsin treatment. Its conformation was unchanged as indicated here by the aurovertin-binding data, and previously by the evidence that uncoupled ATPase activity, competitive inhibition by ADP and noncompetitive inhibition by the inhibitor protein were unchanged [2]. (ATPase activity and F_1-inhibitor binding are thought to be functions of the β-subunit of soluble F_1; see Refs. 15 and 16.) There seemed to be no correlation between the reversible binding of ADP at 40 μM or lower concentration (unchanged, Fig. 2) and the slower sequestration of ADP in the very tight irreversible sites (abolished [2,3]). The reversible sites may actually be on the α-subunit in beef heart F_1 [17,18] but they do not seem to be affected by trypsin cleavage.

It is interesting to compare the α-subunits of beef heart and *E. coli* F_1. In amino acid composition they are extremely similar [6,9,19]. Trypsin cleavage removes a short N-terminal fragment from *E. coli* F_1, which leads to loss of ability to bind to the membrane [20]. Loss of membrane-binding ability is not seen in trypsin-treated beef heart F_1, but there are two proteins in beef heart H^+-ATPase, termed OSCP (oligomycin sensitivity-conferring protein) and F_6, which are responsible for binding F_1 to the membrane [15] and which do not seem to have counterparts in *E. coli* (Senior, unpublished data). Therefore, the α-subunit may not be involved in binding F_1 to the membrane in mitochondrial ATPase. The isolated *E. coli* F_1 α-subunit binds ATP and ADP very tightly, apparently even in EDTA-containing buffer [19,21] and ATP induces a conformational change in the α-subunit [19,21]. The rate of dissociation of ATP from the α-subunit is abnormally slow [19]. It has been proposed [19] that the α-subunit in *E. coli* F_1 normally is bound to nucleotide in the enzyme. The data presented here support this hypothesis for the beef heart enzyme. Cleavage of the α-subunit by trypsin releases all the tightly bound nucleotide, which cannot be rebound [3] even though reversible ADP-binding sites, ATPase sites and all other subunits are intact. It seems likely that an α-subunit nucleotide complex (ATP or ADP) is the functional form of the α-subunit. The irreversibly bound tight nucleotides would then not have a catalytic role. Recent data of Gresser et al. [22] demonstrated that although there are transiently tightly bound nucleotides occurring on F_1 during ATP synthesis which could be intermediate species in the catalysis, they are not the same as the tightly bound nucleotides described earlier [15,19] which copurify with soluble F_1.

The detailed nature of the cleavage of the α-subunit by trypsin is now under further study. As a limited chemical modification which abolishes the overall energy-transducing capability of H^+-ATPase while leaving some partial functions intact, it promises to yield more information regarding the molecular mechanism of action of the complex. In a recent paper, Pedersen et al. [23] showed that trypsin cleavage also abolishes energy-transducing ability in rat liver F_1, although the subunit affected is not yet defined in that enzyme.

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