SELECTIVE PARTIAL DIGESTION OF THE α SUBUNIT OF SOLUBLE MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE (F_1) BY TRYPSIN

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Received 25 August 1981

1. Introduction

Trypsin treatment of soluble mitochondrial ATPase (F_1) from beef heart has been shown to result in a release of firmly bound adenine nucleotides and a concomitant loss of coupling factor activity, while the ATPase activity of the enzyme and its ability to rebind to F_0 and acquire oligomycin sensitivity were unchanged [1]. SDS–polyacrylamide gel electrophoresis revealed no noticeable change in the subunit composition of the enzyme. Similar findings were reported for F_1 from rat liver [2] except that the amount of bound adenine nucleotide in the trypsin-treated enzyme was unaltered and that two new peptides appeared comparable in size to the δ subunit.

Here we show that treatment of soluble F_1 from beef heart mitochondria with low concentrations of trypsin results in a selective, partial digestion of the α subunit, rendering it similar in size to the β subunit. The enzyme so modified exhibits unaltered ATPase activity, and is capable of rebinding to F_0 and acquiring oligomycin sensitivity. It is also capable of supporting ATP-driven transhydrogenase activity and of interacting with the ATPase inhibitor protein.

2. Materials and methods

Mitochondrial ATPase (F_1) was purified from beef heart mitochondria according to [3]. The specific activity was 80–100 μmol . min^{-1} . mg $protein^{-1}$. The enzyme was stored as a 50% saturated $(NH_4)_2SO_4$ suspension at 4°C. Before use, the suspension was spun down, and the sediment was dissolved in a medium consisting of 100 mM Tris-acetate (pH 8) and 2 mM EDTA to 1 mg protein/ml. The solution was passed through a 7 × 0.5 cm Sephadex G-25 column equilibrated with the same medium. ATPase activity was assayed by coupling the reaction to the pyruvate kinase and lactate dehydrogenase systems measuring the oxidation of NADH spectrophotometrically at 340 nm at 30°C.

Trypsin treatment was done by incubating 100 μg F_1 in a medium containing 0.5 μg trypsin, 100 mM Tris-acetate (pH 8) and 2 mM EDTA at 30°C in a final volume of 0.2 ml. After 5 min incubation, trypsin inhibitor (2.5 μg) was added. Since it is possible that trypsin can be reactivated in the presence of SDS due to dissociation of the trypsin–trypsin inhibitor complex, additional controls were run in which trypsin inhibitor was replaced with 3 mM PMSF. Identical results were obtained with both inhibitors.

Oligomycin-sensitive ATPase was tested by incubating F_1 with F_1-depleted submitochondrial particles (ESU particles) in a medium containing 100 mM sucrose, 60 mM Tris-acetate (pH 8) and 1 mM EDTA for 30 min at 25°C. ESU particles were prepared from beef heart mitochondria by treatment of 'EDTA particles' [4] with Sephadex and urea as in [5]. Aliquots were taken and ATPase activity was determined in the absence and presence of 5 μg oligomycin.

Transhydrogenase activity was assayed at 30°C in a medium containing 50 mM Tris-acetate (pH 7.5), 170 mM sucrose, 0.1 mM NADH, 0.2 mM $NADP^+$, 150 μg alcohol dehydrogenase, 8 mM hydrazine, 75 mM ethanol, 1.7 μM rotenone, 5 mM $MgCl_2$, 3 mM

Abbreviations: F_0 and F_1, proton-translocating and catalytic moieties of the mitochondrial ATPase system; ESU particles, submitochondrial particles prepared in the presence of EDTA and treated with Sephadex and urea; M_r, relative molecular mass; NEM, *N*-ethylmaleimide; OSCP, oligomycin-sensitivity conferring protein; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecylsulfate; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

ATP and 0.3 μg oligomycin/mg ESU particle protein, in a final volume of 3 ml. The reduction of $NADP^+$ was followed spectrophotometrically at 340 nm. The reaction rates were corrected for the non energy-linked transhydrogenase activity measured prior to the addition of ATP.

ATPase inhibitor protein was prepared according to [3]. Binding of the inhibitor to F_1 was done by incubation of 13 μg F_1 and 10 μg inhibitor in the presence of 250 mM sucrose, 15 mM Tris–Tes buffer (pH 6.5) and 0.5 mM ATP-Mg^{2+} in a final volume of 50 μl for 20 min at 25°C.

Cold-exposure of F_1 was done in a medium containing 100 mM Tris-acetate (pH 8), 2 mM EDTA and 1 mg F_1/ml in an ice bath for 16 h. The enzyme so treated had a residual activity of <5%.

SDS–polyacrylamide gel electrophoresis was done according to [6] using 15% polyacrylamide containing 0.4% *N,N'*-bis-methylene–acrylamide. Treatment of F_1 with ^{14}C-NEM was done according to [7] with the exception that SDS was not included in the incubation medium and the labelled samples were not treated with ethanol.

3. Results and discussion

Fig.1 shows SDS–polyacrylamide gels of native and trypsin-treated F_1 (lanes B,F). It may be seen that treatment with 5 μg trypsin/mg F_1 for 5 min at 30°C resulted in a striking change in the position of the α band, which moved closer to the β band and virtually fused with the latter. The β band itself as well as the γ and δ bands, remained unchanged; the ε subunit was not visible under the conditions employed. A double band between the γ and δ bands, which was a contaminant in this particular F_1 preparation (probably consisting of firmly bound OSCP [8]) disappeared as a result of the trypsin treatment. Addition of 2 mM ADP or ATP (lanes G,H) 10 min before the trypsin treatment had no effect on the digestion pattern. Trypsin treatment of cold-inactivated F_1 (lanes A,C) led to an extensive digestion of the α as well as the β and γ subunits. Again, ADP and ATP had no effect (lanes D,E). These results show that the β and γ subunits are efficiently protected against trypsin digestion by the quaternary structure of the native enzyme but become accessible to trypsin upon dissociation in the cold; as reported in [8], cold-exposure results in a dissociation of α and/or β subunits

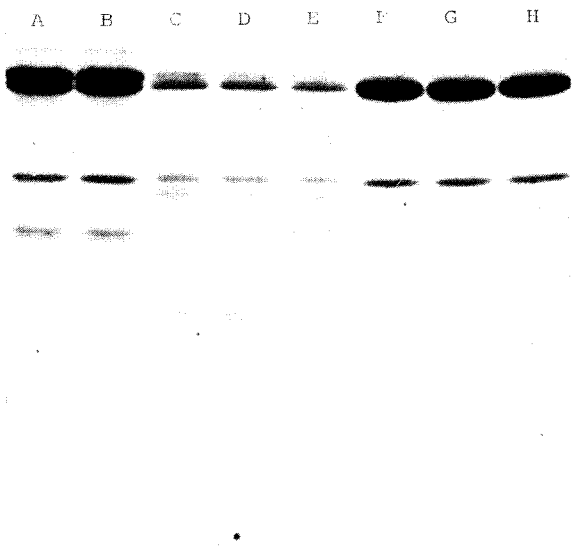

Fig.1. Coomassie brilliant blue staining of SDS–polyacrylamide gel of native and cold-inactivated F_1 before and after treatment with trypsin: (A) cold-inactivated F_1 before trypsin treatment; (B) native F_1 before trypsin treatment; (C) cold-inactivated F_1 after trypsin treatment; (D) C + 2 mM ADP; (E) C + 2 mM ATP; (F) native F_1 after trypsin treatment; (G) F + 2 mM ADP; (H) F + 2 mM ATP. For other details see section 2.

from the enzyme. These results show furthermore that the trypsin attack on the α subunit of the native enzyme is probably limited to one single site and results in the removal of a segment of 2000–3000 M_r from the C- or N-terminus of the α subunit.

That the selective effect of trypsin on the native enzyme concerned the α subunit could be verified with [^{14}C]NEM-labelled F_1 (fig.2). In accordance with [7,9], NEM bound to the α and γ subunits but not the β subunit of F_1 (lane B). Trypsin treatment of the native enzyme (lane F) caused a movement of the radioactivity associated with the α subunit to a somewhat lower M_r position, similar to that seen in the SDS gels in fig.1 and corresponding to the approximate position of the β subunit. The position of the radioactivity of the γ subunit remained unchanged. ADP and ATP (lanes G,H) had no effect. It may be noticed that the total radioactivity in the trypsin-digested α subunits (lanes F–H) was less than in the α subunit of the native enzyme (lane B), suggesting

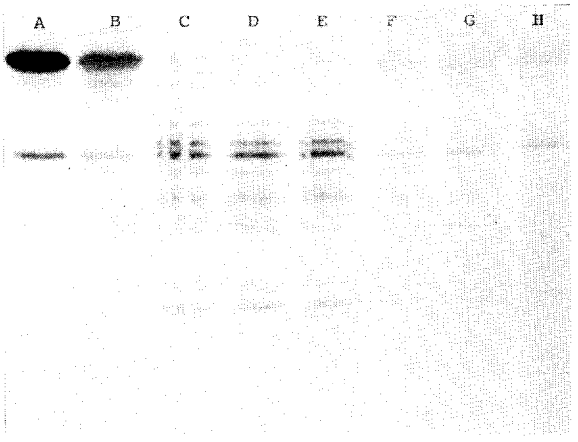

Fig.2. Autoradiograph of SDS–polyacrylamide gel of [^{14}C]-NEM-treated native and cold-inactivated F_1 before and after treatment with trypsin: (A) cold-inactivated F_1 before trypsin treatment; (B) native F_1 before trypsin treatment; (C) cold-inactivated F_1 after trypsin treatment; (D) C + 2 mM ADP; (E) C + 2 mM ATP; (F) native F_1 after trypsin treatment; (G) F + 2 mM ADP; (H) F + 2 mM ATP. For other details see section 2.

that the piece cleaved off by trypsin (which most probably ran off the gels) contained some of the bound NEM, i.e., that this piece may contain some of the sulfhydryl groups of the α subunit. The cold-inactivated enzyme (lane A) was again extensively digested by trypsin both in the absence and presence of ADP or ATP (lanes C–E) and the radioactivity was spread over a number of bands. It may also be noticed that cold-inactivated F_1 (lane A) bound more NEM than did the native enzyme (lane B), indicating that cold-dissociation renders the enzyme more readily accessible to NEM.

Table 1 compares some catalytic parameters of native F_1 before and after treatment with trypsin under the conditions described above. The ATPase activity was unaffected by the trypsin treatment, in accordance with [1,2]. Likewise, the ability of the enzyme to bind to F_1-depleted submitochondrial particles and to acquire oligomycin sensitivity was virtually unaffected. Trypsin also did not abolish the ability of the enzyme to support ATP-driven transhydrogenase activity when rebound to F_1-depleted particles, even though this ability was somewhat diminished by trypsin in comparison with the untreated enzyme. The trypsin-treated enzyme also retained its ability to interact with the ATPase inhibitor of [10]. The ability of the trypsin-treated F_1 to support ATP-driven transhydrogenase is in contrast to [1,2] according to which the trypsin-treated enzyme is no longer capable of functioning as a coupling factor for various energy-linked functions. The reason for this discrepancy may be that the various energy-linked functions in [1,2] were assayed in the absence of oligomycin. In our case, a low concentration of oligomycin was necessary to obtain ATP-driven transhydrogenase activity, in accordance with [11,12].

Table 1
Effect of trypsin treatment on various catalytic parameters of F_1

Parameter	Trypsin treatment	
	Before	After
ATPase activity of soluble F_1 (μmol . min^{-1} . mg protein^{-1})	96	96
Oligomycin sensitivity of F_1 after binding to ESU particles (%)	90	75
ATP-driven transhydrogenase activity (nmol . min^{-1} . mg protein^{-1})	79	48
Inhibition of soluble F_1 by ATPase inhibitor protein (%)	75	90

Trypsin treatment and assay of the various parameters were done as in section 2

These results indicate that soluble F_1 from beef heart mitochondria contains a unique trypsin-sensitive site at which a polypeptide of 2000–3000 M_r can be cleaved off from the C- or N-terminus of the α subunit of the enzyme. Removal of this segment of the α subunit does not influence the ATPase activity of F_1 or its ability to acquire oligomycin sensitivity. The modified enzyme is also capable of supporting ATP-driven transhydrogenase when bound to F_1-depleted submitochondrial particles and of interacting with the ATPase inhibitor protein. Dissociation of F_1 upon cold-exposure opens multiple sites of attack for trypsin on several subunits of F_1.

Acknowledgements

This work has been supported by the Swedish Natural Science Research Council and the Swedish Cancer Society.

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