Limited proteolysis of pig liver CoA synthase: evidence for subunit identity

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The bifunctional enzyme CoA synthase can be nicked by trypsin without loss of its activities. The original dimer of subunit M_r approx. 61 000 yields fragments of M_r 41 000 and 22 000 as seen on gel electrophoresis in the presence of SDS, but the nicked enzyme retains the native M_r of 118 000. Further proteolysis occurs rapidly in the absence of protecting substrates. The N-terminal of native CoA synthase is proline, and proteolysis exposes glycine as a second N-terminal. This evidence strongly suggests that the subunits are identical.

CoA synthase *Limited proteolysis* *N-terminal analysis* *Subunit identity*

1. INTRODUCTION

CoA synthase is a bifunctional enzyme containing phosphopantetheine adenylyltransferase (EC 2.7.7.3) and dephospho-CoA kinase (EC 2.7.1.24) activities [1,2]. It has been purified to homogeneity from pig liver [2], and shown to be a dimer, both subunits having an M_r of approx. 61000. No evidence was found for channeling of dephospho-CoA between the two catalytic sites [3].

Limited proteolysis has proved a useful tool in studying the structure of multifunctional proteins and in many cases has provided a method of isolating functional domains [4–6]. Here, limited proteolysis is used in conjunction with N-terminal analysis to investigate the identity of the CoA synthase subunits.

Abbreviations: TPCK, 1-tosylamide-2-phenylethyl chloromethyl ketone; DABITC, dimethylaminobenzene-4-isothiocyanate; PITC, phenylisothiocyanate

2. MATERIALS AND METHODS

2.1. *Materials*

CoA was obtained from PL Biochemicals (USA). TPCK-treated trypsin and trypsin-inhibitor agarose were from Sigma (Poole, England). DABITC and PITC were obtained from BDH (England).

4′-Phosphopantetheine was prepared according to [7], and purified by DEAE-cellulose chromatography [8].

CoA synthase was purified from pig liver as in [2], except that it was stored at −20°C after CoA affinity elution and passed through Sephadex G-150 prior to use.

2.2. *Enzyme assays*

Phosphopantetheine adenylyltransferase was assayed in the reverse direction of ATP production as in [2]. The kinase activity and the complete CoA synthase reaction were assayed by measuring ADP production [2], using dephospho-CoA or 4-phosphopantetheine as substrates, respectively.

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2.3. *Electrophoresis*

Polyacrylamide gel electrophoresis in the presence of SDS was carried out in 12.5% slab gels at pH 8.5 [9], and proteins were stained with Coomassie blue.

2.4. *Proteolysis*

CoA synthase (0.3 mg/ml) was incubated with TPCK-treated trypsin (1.5 μg/ml) at room temperature in 50 mM Tris-HCl, pH 8, in the presence and absence of 0.5 mM CoA. The enzyme was assayed at 5-min intervals, and 20-μg samples were precipitated with 6% (w/v) trichloroacetic acid at 10 and 20 min for gel electrophoresis. 2 mM MgATP and 0.5 mM 4'-phosphopantetheine were also tested as protecting agents.

2.5. *Gel filtration*

Enzyme (0.1 mg) was incubated at 4°C with 0.5 μg TPCK-treated trypsin in 0.5 mM CoA, 0.5 mM dithiothreitol, 10 mM Tris-HCl, pH 8, in a final volume of 0.5 ml. After 15 min the reaction mixture was passed through a column (1 × 0.3 cm) of trypsin-inhibitor agarose. Gel filtration of a mixture of native CoA synthase (0.1 mg) and this nicked enzyme was carried out using a Sephadex G-150 column (35 × 1 cm) in 50 mM Tris-HCl, pH 8, containing 0.5 mM dithiothreitol.

2.6. *N-terminal analysis*

This was performed by the manual liquid-phase DABITC/PITC method described in [10], using TLC analysis to identify DABTH amino acids.

3. RESULTS AND DISCUSSION

Exposure of CoA synthase to small amounts of trypsin in the presence of CoA causes rapid disappearance of the 61 kDa subunit band, yielding two new fragments with approximate molecular masses of 41 and 22 kDa (fig.1). The nicked enzyme is still capable of producing CoA from ATP and phosphopantetheine (fig.2), and when assayed separately, the transferase and kinase retain a constant ratio of activities before and after tryptic digestion. Further proteolysis of the fragments (particularly the 41 kDa fragment) is protected by CoA and to a lesser extent by ATP and

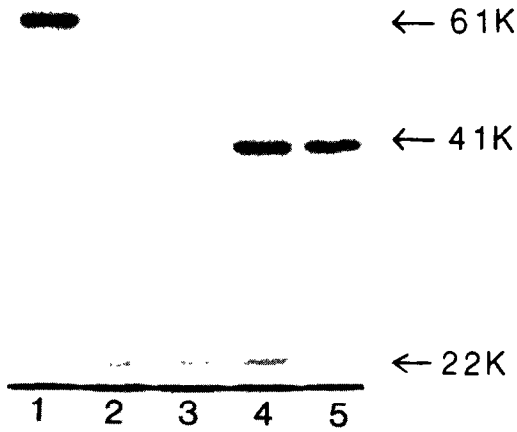

Fig.1. Cleavage of CoA synthase with trypsin as described in section 2.4; 12.5% polyacrylamide gel electrophoresis of 20-μg samples in the presence of SDS. Track 1, enzyme before incubation; tracks 2 and 3, 10 and 20 min samples from incubation with trypsin and no CoA; tracks 4 and 5, 10 and 20 min samples of incubation in the presence of 0.5 mM CoA.

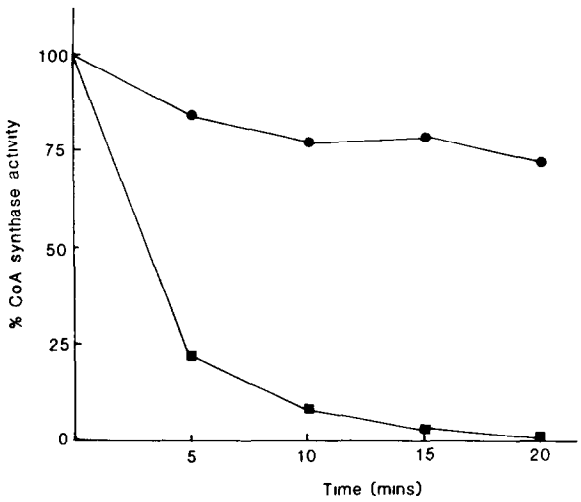

Fig.2. Decrease in CoA synthase activity on incubation with trypsin in the presence (●) and absence (■) of 0.5 mM CoA. The enzyme was assayed for the production of ADP from phosphopantetheine and ATP.

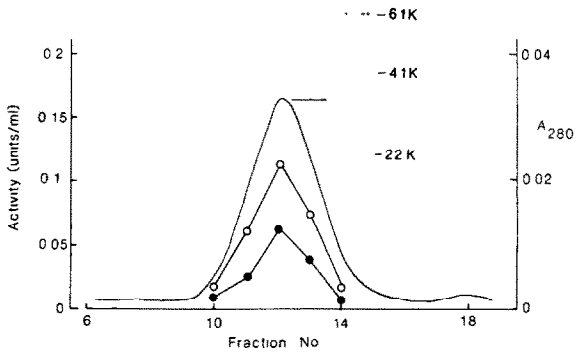

Fig.3. Sephadex G-150 gel filtration of native and trypsin-treated enzyme. Fractions (0.8 ml) were assayed for both transferase (○) and kinase (●) and the peak fraction was subjected to 12.5% polyacrylamide gel electrophoresis in the presence of SDS.

phosphopantetheine. A similar pattern of digestion was also obtained using subtilisin.

Gel filtration of a mixture of native and trypsin-treated enzyme gave a single peak showing all 3 polypeptide bands (fig.3), indicating that the nicked enzyme has retained the native M_r of approx. 118000. It would therefore appear that this cleavage has not disrupted the quaternary structure of the protein, which explains why the activities are preserved. Attempts were made to denature and reanneal CoA synthase using 8 M urea and 6 M guanidinium chloride, with a view to separating the fragments under such conditions, and examining their activity. However, we were unable to restore lost activities, and the possibility of separable domains remains to be seen.

This apparent single cleavage point is in itself strong evidence for an α_2 rather than an $\alpha\beta$ subunit structure for CoA synthase. N-terminal analysis of the native enzyme was found to give a single proline N-terminal, and nicking with trypsin (subsequently removed on trypsin-inhibitor agarose) exposed glycine as a new N-terminal residue. These results strongly suggest that CoA synthase contains two identical subunits.

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