Proteolysis of *Acinetobacter* citrate synthase by subtilisin

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Received 15 November 1982

Citrate synthase from *Acinetobacter calcoaceticus* was subjected to proteolysis with subtilisin. Although the enzyme proved relatively resistant to inactivation by this treatment, SDS–polyacrylamide gel electrophoresis clearly revealed breakdown of the citrate synthase to smaller fragments. The regulatory responses of the native enzyme to inhibition by NADH and re-activation by AMP were retained on proteolysis, indicating that the fragments bind tightly to each other and preserve the overall cooperative molecular interactions.

Citrate synthase Acinetobacter calcoaceticus *Proteolysis* *Subtilisin* *Cooperativity* *Regulation*

1. INTRODUCTION

Many cases of the proteolysis of enzymes resulting in the production of discrete fragments have been described and several categories of effects may be identified. First there are those cases in which no enzyme activity is lost, e.g. with ribonuclease [1]. Another group contains those enzymes which do lose activity on proteolysis, as in the cases of citrate synthase from pig heart [2] and glutamine synthetase from *Staphylococcus aureus* [3]. A further group consists of enzymes whose catalytic activity is actually enhanced by proteolysis, e.g., fructose 1,6-bisphosphatase from rabbit liver [4]. In all these examples, the peptide fragments are held together strongly by non-covalent interactions, the molecular size of the enzyme after proteolysis being similar to that of the native form. However, the kinetic properties may be altered, as in the case of pig heart citrate synthase [2].

In this communication we report that treatment of citrate synthase from the bacterium *Acinetobacter calcoaceticus* with the proteolytic enzyme subtilisin results in complete loss of the native enzyme subunit. This is accompanied by a small loss of activity but the regulatory properties of the native enzyme are essentially retained.

2. EXPERIMENTAL

Subtilisin–BPN and phenylmethylsulphonyl fluoride (PMSF) were obtained from Sigma. *A. calcoaceticus* citrate synthase (EC 4.1.3.7) was purified and assayed as in [5].

Proteolysis of citrate synthase was carried out in 20 mM triethanolamine–HCl buffer (pH 8.0) (TEA-8) at 25°C with either 0.84 or 2.08 mg of citrate synthase and the same weight of subtilisin, in a total reaction volume of 1 ml. The reaction was initiated by the addition of the subtilisin. For activity studies, samples were removed and diluted 200-fold with TEA-8; this prevented further loss of activity even up to 20 h after dilution. For electrophoretic analysis, samples of the reaction mixture containing 15–20 μg of citrate synthase protein were added to 200 μl of TEA-8 containing 1 mM PMSF and incubated for 15 min at 4°C. After drying down under vacuum, electrophoresis was performed without removal of excess reagents.

Non-denaturing polyacrylamide gel electro-

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Published by Elsevier Biomedical Press
00145793/83/0000—0000/$3.00

phoresis (PAGE) [6] and sodium dodecyl sulphate PAGE (SDS–PAGE) [7] were performed as in [5].

Gel filtration was carried out on a column (2.5 × 30 cm) of Sephadex G-200 equilibrated with buffer containing 20 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$ and 1 mM EDTA.

3. RESULTS AND DISCUSSION

Acinetobacter citrate synthase proved to be very resistant to inactivation by the proteolytic enzyme subtilisin. At a ratio of 1:1 (w/w), between zero and 16% activity was lost (in different experiments) after incubation for 5 h (fig. 1). However, after 7 h about 80% activity was lost, and incubation for 17 h resulted in almost total inactivation of the enzyme.

These results are in complete contrast to those reported for the citrate synthase of pig heart [8] in which case full inactivation was effected by subtilisin within 1 h at a lower protease to enzyme ratio of 1:100 (w/w). Moreover, proteolysis of the pig enzyme was reported to be absolutely dependent on the presence of palmitoyl–CoA. We found that palmitoyl–CoA (4.62 μM; 5 mol/mol of citrate synthase subunit) did not enhance the rate of inactivation of the *Acinetobacter* enzyme. In fact a slight protection against inactivation was observed. Similar results were obtained using the proteolytic enzymes trypsin and chymotrypsin.

This difference in susceptibility of the two citrate synthases to inactivation by subtilisin may be related to the difference in enzyme size and complexity. The *Acinetobacter* enzyme is a hexamer of M_r ~250000 whereas the pig heart enzyme is a dimer of M_r 98000. It could be that subtilisin acts at a site remote from the active site of the bacterial enzyme. On the other hand, the relatively small loss of activity during the first 5 h of reaction may suggest that proteolysis does not occur to any sizeable extent during this period.

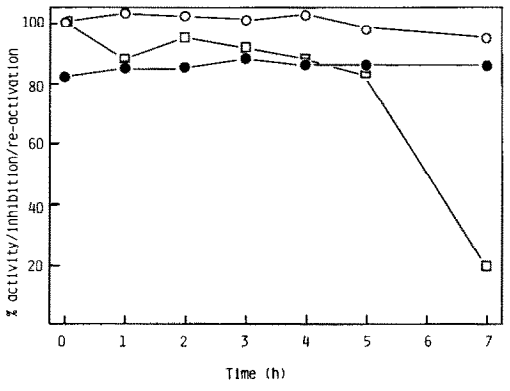

Fig.1. Time dependence of the effects of subtilisin on *Acinetobacter* citrate synthase: (□) enzyme activity; (●) inhibition by 0.2 mM NADH; (○) re-activation by 0.2 mM AMP in the presence of 0.2 mM NADH.

We therefore analysed the reaction products by SDS–PAGE in order to examine the extent of any proteolytic breakdown. The results showed that the native enzyme subunit is indeed broken down by subtilisin. Significant loss of the native subunit occurred in the first 5 h and was complete after 7 h of reaction (fig.2). In place of the native subunit the major bands revealed on SDS–PAGE correspond to fragments of M_r 34000 and 14000, with additional intermediate bands around 29000 and 23000. Smaller fragments of 8000 and 6000 were also observed but these were usually masked by the fragments of subtilisin itself.

Surprisingly, the sensitivity of the enzyme to inhibition by NADH and reactivation by AMP (regulatory features of the native enzyme) remained constant over the entire 7-h reaction of subtilisin with the enzyme (fig.1). This prompted us to investigate the NADH and AMP dependences of enzyme which had been proteolysed by subtilisin to the point at which there was no longer any native subunit (fig.3). The responses to both NADH and AMP resembled those of the native enzyme in being sigmoidal. However, the proteolysed enzyme was less sensitive to NADH inhibition and more sensitive to AMP reactivation than was the native citrate synthase. Similar observations were made in the case of proteolysis with trypsin (not shown).

The possibility existed that proteolysis occurred after samples had been added to the dissolving buffer for SDS–PAGE. Citrate synthase may have been denatured much faster than subtilisin, thus increasing its own susceptibility to proteolytic attack. That this was not the case was demonstrated by first passing a sample of reaction mixture through a column of Sephadex G-200 to separate citrate synthase from the much smaller subtilisin. Subsequent SDS–PAGE revealed a pattern of fragments similar to that found when subtilisin was not removed. The regulatory behaviour towards

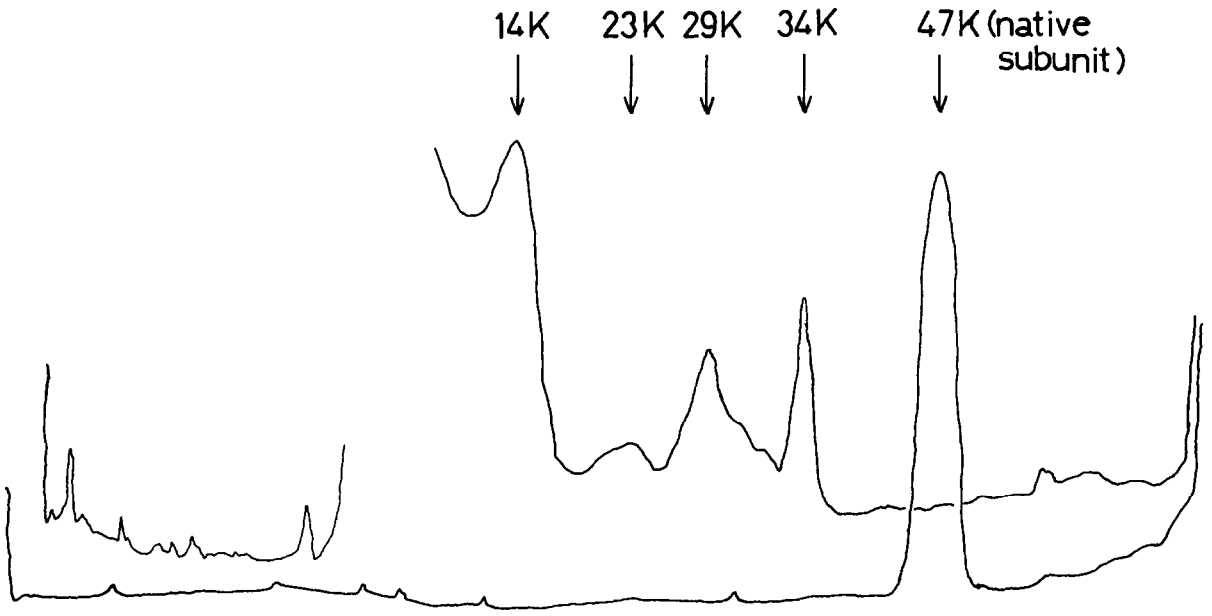

Fig.2. SDS–PAGE (10% polyacrylamide) of native (lower trace) and proteolysed (upper trace) citrate synthase. Fifty μg of citrate synthase protein were applied to each gel. Subtilisin was not removed before electrophoresis. The tops of the gels are to the right of the traces.

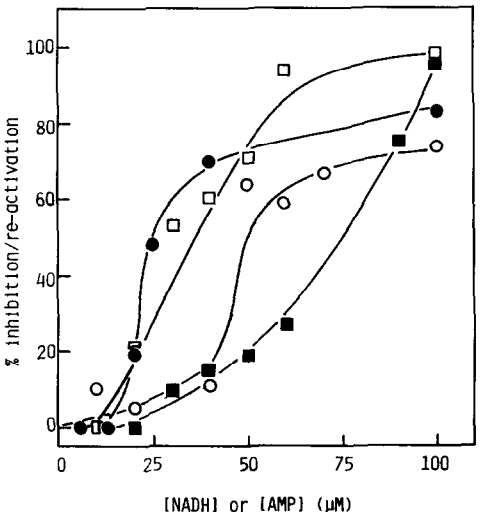

Fig.3. NADH inhibition and AMP re-activation of native and proteolysed citrate synthase. After proteolysis, citrate synthase was separated from subtilisin by gel filtration on Sephadex G-200: (●) NADH inhibition of native enzyme; (○) NADH inhibition of proteolysed enzyme; (■) AMP re-activation of native enzyme; (□) AMP re-activation of proteolysed enzyme. In all cases, AMP re-activation was measured in the presence of 0.2 mM NADH.

NADH and AMP was also the same.

It is thus evident that although no native citrate synthase subunit remained after proteolysis, the fragments bind tightly to each other and thereby fully retain the cooperative interactions characteristic of the native enzyme.

The molecular sizes of the native and proteolysed forms of the enzyme were compared by gel filtration on Sephadex G-200 (fig.4). Both forms were eluted from the column in exactly the same position with respect to the marker, lactate dehydrogenase. Furthermore, non-denaturing PAGE showed that both forms of the enzyme had a relative mobility of 0.24. Thus by these two techniques no difference in size was detectable. The proteolysed enzyme also resembled the native enzyme in kinetic characteristics. The K_m values for oxaloacetate and acetyl–CoA were determined to be 3.6 μM and 142 μM for the native enzyme and 3.8 μM and 99 μM for the proteolysed enzyme.

We are currently studying the proteolysis of citrate synthase by trypsin and chymotrypsin and are attempting to resolve the products of proteolysis into single components in order to ascer-

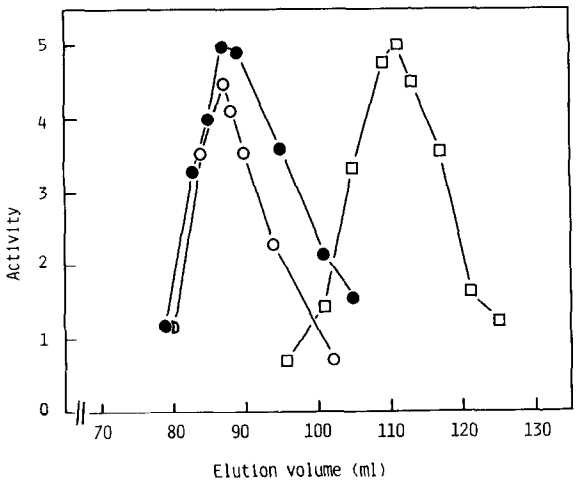

Fig.4. Elution profiles of native and proteolysed citrate synthase from a column of Sephadex G-200: (□) Lactate dehydrogenase; (●) native citrate synthase; (○) proteolysed citrate synthase. Activities are in arbitrary units. See section 2 for further details.

tain whether activity and regulatory properties can be attributed to particular fragments or require their complete assembly.

ACKNOWLEDGEMENT

We thank the Science and Engineering Research Council for support (Grant GR/A/82857).

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