Models of proteolysis of oligomeric enzymes and their applications to the trypsinolysis of citrate synthases

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A simple statistical approach was used to generate predictive models of the proteolysis of multisubunit enzymes in order to correlate the loss of enzyme activity with the loss of native subunit. The models were applied to the trypsinolysis of the citrate synthases of pig heart, *Bacillus megaterium* and *Escherichia coli*. With the dimeric citrate synthases (pig heart and *B. megaterium*) trypsinolysis of one of the subunits appears to destroy the activity of the whole enzymic molecule. The hexameric *E. coli* citrate synthase behaves like a trimer of dimeric units, each of the dimers behaving similarly to the *B. megaterium* and pig heart enzymes. Palmitoyl-CoA is required for the trypsinolysis of pig heart citrate synthase, and at relatively high concentrations of this compound trypsinolysis of one subunit leaves the other subunit fully active. Palmitoyl-CoA is not required for the trypsinolysis of the other citrate synthases, and high concentrations of this metabolite do not affect the correlation of proteolysis with inactivation of these enzymes.

INTRODUCTION

Citrate synthase (EC 4.1.3.7) catalyses the reaction:

The regulatory properties of the enzyme have been found to correlate with the nature and arrangement of its constituent subunits [reviewed by Weitzman & Danson (1976), Weitzman (1981) and Danson (1988)]. Thus the citrate synthases from all eukaryotes and Gram-positive bacteria are dimeric enzymes, subject to isosteric inhibition by ATP. On the other hand, hexameric citrate synthases are found in the Gram-negative bacteria and these enzymes are subject to allosteric control by NADH. Evidence that the allosteric inhibitions are a function of the hexameric nature of the enzyme has come from studies of mutant citrate synthases (Harford & Weitzman, 1978; Danson *et al.*, 1979).

In the present paper we have chosen to study this correlation between subunit arrangement and catalytic and regulatory function by using limited proteolysis. This technique can yield detailed information about conformational movements and about domain and subunit interactions. To this end, predictive models of the effects of proteolytic digestion can be an invaluable aid in the interpretation of data. We describe a statistical approach to predicting the correlation between the rate of loss of native protein (as judged by SDS/polyacrylamide-gel electrophoresis) and the rate of loss of enzyme activity during proteolytic digestion of the enzyme. This approach generates a set of models differing in the effects of proteolysis of one subunit of an oligomeric enzyme on the other subunits and on the activity of the whole enzyme. Data can then be compared with the different models and the best-fitting model selected. This approach was used in the analysis of the trypsinolysis of citrate synthases from pig heart, *Bacillus megaterium* and *Escherichia coli*; these enzymes were chosen as representa-

tives of eukaryotic citrate synthases and of the dimeric and hexameric proteins from prokaryotic organisms.

EXPERIMENTAL

Materials

Chemicals used were of analytical grade or the finest grade commercially available. CoA, oxaloacetate and pig heart citrate synthase were from Boehringer (Mannheim, Germany). 5,5'-Dithiobis-(2-nitrobenzoic acid), trypsin and soya-bean trypsin inhibitor were from Sigma Chemical Co. (Poole, Dorset, U.K.).

B. megaterium D101 was from the culture collection of P. D. J. W., and *E. coli* DB1002 (Bloxham *et al.*, 1983) was a gift from Dr. D. P. Bloxham.

Assay of citrate synthase

Citrate synthase was assayed spectrophotometrically at 412 nm and 25 °C by the method of Sreere *et al.* (1963). Assay mixtures contained 20 mM-Tris/HCl buffer, pH 8.0, 1 mM-EDTA, 0.1 mM-oxaloacetate, 0.15 mM-acetyl-CoA and 0.1 mM-5,5'-dithiobis-(2-nitrobenzoic acid). The reaction, in a final volume of 1.0 ml, was started with enzyme.

Purification of citrate synthase

B. megaterium citrate synthase was purified by the method of Robinson *et al.* (1983). *E. coli* citrate synthase was purified by the method of Weitzman (1969).

Preparation of palmitoyl-CoA

Palmitoyl-CoA was prepared by the method of Chase & Tubbs (1972). The concentration of palmitoyl-CoA was determined from its absorbance at 260 nm (ϵ_{260} $16.4 \times 10^6 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

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Polyacrylamide-gel electrophoresis

Electrophoresis was performed in the presence of SDS in 10% polyacrylamide gels by the procedure of Weber & Osborn (1969). The gels were stained with Coomassie Brilliant Blue R, and the staining was quantified by using a gel scanner (ISCO model 1310) with an LOC/Milton Roy CI-10 integrator. In control experiments the integrated area of the peak obtained was linear with respect to the amounts of citrate synthase up to at least 25 μ g of protein, thereby demonstrating the equal recovery of protein from each sample applied to the gels.

Trypsinolysis

Citrate synthase (3.5 mg of protein/ml) and trypsin were mixed at 25 °C in 1 mM-EDTA/20 mM-Tris/HCl buffer, pH 8.0 [20% (v/v) glycerol was also included with *B. megaterium* citrate synthase to stabilize the enzyme at 25 °C], to give a citrate synthase/trypsin weight ratio of 100:1. At recorded times, 60 μ l of the reaction mixture was taken and added to 100 μ l of 1 mM-EDTA/20 mM-Tris/HCl buffer, pH 8.0, containing 0.5 μ g of soya-bean trypsin inhibitor to stop further proteolysis. Samples of this 'stopped' mixture were then assayed for citrate synthase activity and were subjected to SDS/polyacrylamide-gel electrophoresis.

RESULTS

Stochastic model of the effects of limited proteolysis on a hexameric enzyme

The aim of this analysis is to describe the expected correlation between the activity and amount of the native form of an oligomeric enzyme upon its limited proteolysis. The equations were formulated for a hexameric enzyme because the *E. coli* citrate synthase has that subunit arrangement (Tong & Duckworth, 1975); it is then a simple matter to adapt the equations to the dimeric enzymes of *Bacillus* and pig heart.

We consider a hexameric molecule composed of identical subunits (a to f), each of which can exist in two states, native and proteolysed, the latter having the possibility of an infinite number of substates. It is assumed that native subunits can be separated from proteolysed polypeptides and in the experimental part of this paper we have used SDS/polyacrylamide-gel electrophoresis and subsequent gel scanning to achieve this. It is also assumed that all of the subunits are equally prone to proteolysis and that this condition is unaffected by proteolysis of neighbouring subunits. Finally, it is assumed in the analysis that the proteolysed subunits do not possess catalytic activity. The effects of any of these assumptions being incorrect are described in the Discussion section.

During the course of the proteolysis, any hexamer will be in one of seven forms (A to G), that is having zero to six subunits proteolysed respectively. The analysis presented calculates the relative proportions of molecules in each of these forms, i.e. $P(A)$, $P(B)$, $P(C)$, $P(D)$, $P(E)$, $P(F)$ and $P(G)$.

Consider a solution of m subunits (i.e. $m/6$ molecules) in which i subunits have been proteolysed. The total

possible combinations of distributing i among m subunits is given by z , where:

$$z = \frac{m!}{i!(m-i)!} \quad (1)$$

Considering a specific subunit, a, then the subpopulation of z in which subunit a is proteolysed is given by y , where:

$$y = \frac{(m-1)!}{(i-1)!(m-i)!} \quad (2)$$

Similarly:

$$x = \frac{(m-2)!}{(i-2)!(m-i)!} \text{ (a and b proteolysed)} \quad (3)$$

$$w = \frac{(m-3)!}{(i-3)!(m-i)!} \text{ (a, b and c proteolysed)} \quad (4)$$

$$v = \frac{(m-4)!}{(i-4)!(m-i)!} \text{ (a, b, c and d proteolysed)} \quad (5)$$

$$u = \frac{(m-5)!}{(i-5)!(m-i)!} \text{ (a, b, c, d and e proteolysed)} \quad (6)$$

and

$$t = \frac{(m-6)!}{(i-6)!(m-i)!} \text{ (a, b, c, d, e and f proteolysed)} \quad (7)$$

Eqns. (2)–(7) do not define mutually exclusive subpopulations of z . For example, u (eqn. 6) consists of two components, the number in which a–e are proteolysed and the number in which a–f are proteolysed. It is the former quantity that is required and this (u') is obtained by subtraction:

$$u' = u - t \quad (8)$$

Similarly:

$$v' = v - 2u' - t \quad (9)$$

$$w' = w - 3v' - 3u' - t \quad (10)$$

$$x' = x - 4w' - 6v' - 4u' - t \quad (11)$$

$$y' = y - 5x' - 10w' - 10v' - 5u' - t \quad (12)$$

Eqns. (7)–(12) define the subpopulations of z of one subform of each of the forms B to G. However, B to F have multiple subforms depending upon exactly which of their subunits has been proteolysed. In general, for A to G, the number of subforms can be obtained from the formula for the number of combinations (c) of n objects taken r at a time:

$$c = n!/[r!(n-r)!] \quad (13)$$

For A to G the numbers of subforms are 1, 6, 15, 20, 15, 6 and 1 respectively.

To obtain the number in the subpopulation of z for all the subforms, eqns. (7)–(12) are multiplied by the respective number of subforms. Then, the probabilities of obtaining A to G are found by dividing by the total number of possibilities (z):

$$P(G) = t/z \quad (14)$$

$$P(F) = 6u'/z \quad (15)$$

$$P(E) = 15v'/z \quad (16)$$

$$P(D) = 20w'/z \quad (17)$$

$$P(C) = 15x'/z \quad (18)$$

$$P(B) = 6y'/z \quad (19)$$

$$P(A) = 1 - [P(B) + P(C) + P(D) + P(E) + P(F) + P(G)] \quad (20)$$

In terms of i and m , eqns. (14)–(20) can be written:

$$P(A) = 1 - [P(B) + P(C) + P(D) + P(E) + P(F) + P(G)]$$

$$P(B) = \frac{6i!}{m!} \left[\frac{(m-1)!}{(i-1)!} - \frac{5(m-2)!}{(i-2)!} + \frac{10(m-3)!}{(i-3)!} - \frac{10(m-4)!}{(i-4)!} + \frac{5(m-5)!}{(i-5)!} - \frac{(m-6)!}{(i-6)!} \right]$$

$$P(C) = \frac{15i!}{m!} \left[\frac{(m-2)!}{(i-2)!} - \frac{4(m-3)!}{(i-3)!} + \frac{6(m-4)!}{(i-4)!} - \frac{4(m-5)!}{(i-5)!} + \frac{(m-6)!}{(i-6)!} \right]$$

$$P(D) = \frac{20i!}{m!} \left[\frac{(m-3)!}{(i-3)!} - \frac{3(m-4)!}{(i-4)!} + \frac{3(m-5)!}{(i-5)!} - \frac{(m-6)!}{(i-6)!} \right]$$

$$P(E) = \frac{15i!}{m!} \left[\frac{(m-4)!}{(i-4)!} - \frac{2(m-5)!}{(i-5)!} + \frac{(m-6)!}{(i-6)!} \right]$$

$$P(F) = \frac{6i!}{m!} \left[\frac{(m-5)!}{(i-5)!} - \frac{(m-6)!}{(i-6)!} \right]$$

$$P(G) = \frac{i!}{m!} \left[\frac{(m-6)!}{(i-6)!} \right]$$

These equations can now be used to predict the loss of activity during proteolysis, assuming various models of how subunit interactions relate to catalysis. The models considered depend on the known oligomeric nature of the enzyme; in the case of the hexameric citrate synthases, for example, plausible models on symmetry grounds would be: (1) only unproteolysed hexamers are active; (2) the hexamer functions as a dimer of trimers; (3) the hexamer functions as a trimer of dimers; or (4) all six subunits are active independently of their neighbours.

Models (3) and (4) in turn describe the situations for the dimeric citrate synthases, which could be active either only as a dimer or as two independent monomers.

Trypsinolysis of citrate synthases

(a) Pig heart citrate synthase. Trypsinolysis of pig heart citrate synthase initially produced two major fragments of M_r 34000 and 10000, with the secondary appearance of a polypeptide of M_r 30000. These observations are in agreement with the data of Bloxham *et al.* (1980) and, as reported by those workers, the presence of palmitoyl-CoA was found to be necessary for proteolysis. The peptides produced and their relative rates of appearance did not differ between 50 μ M- and 150 μ M-palmitoyl-CoA, although the correlation between loss of catalytic activity and the trypsinolysis of native enzyme showed a marked difference between the two concentrations of thioester (Fig. 1). Applying the analysis detailed above, it is concluded that, at the lower concentration of palmitoyl-CoA, the active unit of the citrate synthase is a dimer, and proteolysis of one subunit destroys the activity of both. However, at the higher concentration, the monomers appear to function independently with each retaining its activity even when its neighbour is proteolysed.

Clearly, increasing the concentration of palmitoyl-CoA between 50 and 150 μ M causes subtle changes in the structure of pig citrate synthase, principally affecting the dependence of catalytic activity on the dimeric unit. The

nature of this structural change is undefined at present, although it is known that the interaction of the acyl-CoA with the enzyme is complex and may include both

inhibition and, at higher concentrations, inactivation of catalytic activity [Else *et al.* (1988) and references cited therein]. At the concentration of citrate synthase used (3.5 mg of protein/ml, which is equivalent to 70 μ M-polypeptide chain), it is unlikely that the protein is

Fig. 1. Trypsinolysis of pig heart citrate synthase: correlation of proteolysis and inactivation

Pig heart citrate synthase was incubated with trypsin as described in the Experimental section. At recorded time intervals the enzymic activity was assayed and samples were removed for SDS/polyacrylamide-gel electrophoresis to assess the degree of proteolysis. The data are plotted as fraction of unproteolysed polypeptide chains versus fraction of enzyme activity remaining: ●, trypsinolysis in the presence of 50 μ M-palmitoyl-CoA; ○, trypsinolysis in the presence of 150 μ M-palmitoyl-CoA. The theoretical curves are those predicted assuming that the minimum subunit structure of the active enzyme is hexameric (a), trimeric (b), dimeric (c) and monomeric (d).

Fig. 2. Trypsinolysis of citrate synthase from *B. megaterium*: appearance of proteolytic fragments

Trypsinolysis was carried out as described in the Experimental section. Samples were removed for SDS/polyacrylamide-gel electrophoresis at recorded time intervals to assess the rates of proteolysis and appearance of the proteolytic fragments: ●, native unproteolysed subunit; ○, fragment of M_r 41000; ▲, fragment of M_r 28000; △, fragment of M_r 27000.

Fig. 3. Trypsinolysis of citrate synthase from *B. megaterium*: correlation of proteolysis and inactivation

The data from Fig. 2 for the native subunit are plotted as fractions of unproteolysed polypeptide chains versus the fraction of enzymic activity remaining. The theoretical curves are those predicted assuming that the minimum subunit structure of the active enzyme is hexameric (a), trimeric (b), dimeric (c), and monomeric (d).

saturated with palmitoyl-CoA, although the observed effect of $150 \mu\text{M}$ of this ligand in the present experiments may reflect the beginning of an unfolding of the protein.

(b) *B. megaterium* citrate synthase. Three main peptides were produced on trypsinolysis of *B. megaterium* citrate synthase, with M_r values of 41000, 28000 and 27000

Fig. 4. Trypsinolysis of citrate synthase from *E. coli*: appearance of proteolytic fragments

Trypsinolysis was carried out as described in the Experimental section. Samples were removed for SDS/polyacrylamide-gel electrophoresis at recorded time intervals to assess the rates of proteolysis and appearance of the proteolytic fragments: ●, native unproteolysed subunit; ○, fragment of M_r 31500; ▲, fragment of M_r 14500; △, fragment of M_r 11500.

(Fig. 2). Minor bands of M_r 22000, 21500 and 16500 could also be observed if excess proteolysed enzyme was applied to the polyacrylamide gels. A plot of the percentage of native subunit versus the percentage of enzymic activity indicated either a dimer or a trimer as the catalytic unit (Fig. 3). *B. megaterium* citrate synthase is a dimeric protein (Robinson *et al.*, 1983) and therefore the choice of catalytic unit within this oligomer is either the individual monomer or the dimer unit itself. From the data in Fig. 3 we conclude that the dimer is required for enzymic activity, as in the case of pig heart citrate synthase. Possible reasons for the data points lying above the curve for the dimeric unit are outlined in the Discussion section.

No requirement of proteolysis for the presence of palmitoyl-CoA was observed, nor did this compound significantly affect the nature or rate of appearance of the peptide products.

(c) *E. coli* citrate synthase. Trypsinolysis of *E. coli* citrate synthase proceeded in a manner similar to that described by Bell *et al.* (1983). Fragments of M_r 31500 and 14500 were produced with time, along with a minor component of M_r 11500 (Fig. 4). Again, analysis of these data showed the catalytically active unit to be a trimer or dimer (Fig. 5). In fact, the plot shown in Fig. 5 coincides precisely with that for the dimeric citrate synthases of *B. megaterium* (Fig. 3) and pig heart (Fig. 1) at low palmitoyl-CoA concentrations. To illustrate the coincidence, the data points for the *Bacillus* and pig heart enzymes are included in Fig. 5 with those for *E. coli*.

The presence of palmitoyl-CoA did not affect the rate of trypsinolysis, as judged by SDS/polyacrylamide-gel electrophoresis, nor did it alter the pattern of fragments observed. The effect of the presence of the thioester on

Fig. 5. Trypsinolysis of citrate synthase from *E. coli*: correlation of proteolysis and inactivation

The data from Fig. 4 for the *E. coli* native subunit (●) are plotted as fractions of unproteolysed polypeptide chains versus the fraction of enzymic activity remaining. The theoretical curves are those predicted assuming that the minimum subunit structure of the active enzyme is hexameric (a), trimeric (b), dimeric (c) and monomeric (d). The corresponding data for the trypsinolysis of pig heart citrate synthase in the presence of 50 μ M-palmitoyl-CoA (Δ), taken from Fig. 1, and those for the *B. megaterium* enzyme ($\square$) from Fig. 2, are included for comparison.

the loss of catalytic activity could not be assessed because the concentration of palmitoyl-CoA needed to cause an effect on the trypsinolysis of pig heart citrate synthase would cause complete inhibition of the *E. coli* enzyme (Else, 1985).

The allosteric inhibition of *E. coli* citrate synthase by NADH remained unaffected by the proteolysis.

DISCUSSION

Although the proteolysis of pig heart and *E. coli* citrate synthases has been reported previously (Bayer *et al.*, 1981; Bloxham *et al.*, 1981, 1982, 1983; Bell *et al.*, 1983; Lill *et al.*, 1984), in the present paper we have quantitatively correlated the effects of the proteolytic cleavages with the consequent inactivations of the enzymes. In addition, we have studied, for the first time, the trypsinolysis of the dimeric citrate synthase from *B. megaterium*. These correlations provide valuable information on the functional significance of the oligomeric nature of these enzymes, and in the analysis of the experimental data we have developed equations that are of general applicability.

Our data strongly indicate that the dimer is the basic catalytic unit of all three citrate synthases, which are representative of eukaryotes and the Gram-positive and Gram-negative bacteria. In the case of the pig heart enzyme, X-ray crystallography shows that each of the two active sites is composed of amino acid residues from both polypeptide chains (Remington *et al.*, 1982). Thus it is quite reasonable to expect that proteolysis of one

subunit would affect both active sites. A similar situation is assumed for the dimeric citrate synthase of *B. megaterium*, consistent with its kinetic and regulatory properties being remarkably similar to those of the eukaryotic enzyme (Weitzman & Danson, 1976; Weitzman, 1981; Robinson *et al.*, 1983).

The hexameric *E. coli* citrate synthase appears functionally to be a trimer of dimers. Consistent with this conclusion are our previous observations with mutants of the *E. coli* enzyme (Weitzman & Danson, 1976; Danson *et al.*, 1979). Thus we have been able to isolate catalytically active mutants of *E. coli* citrate synthase that are of the 'small' type and, although not yet purified, their native M_r values would indicate a dimeric structure.

We have provided evidence that the mutant enzyme is a product of the altered wild-type gene (Danson *et al.*, 1979). It is therefore probable that the normal enzyme is a trimer of dimers and that the mutation has caused a dissociation into the active dimer, with the concomitant loss of its regulatory potential.

It should also be noted that the primary sequence of the *E. coli* citrate synthase is approx. 30% homologous with that of the pig heart enzyme, with several key catalytic residues being conserved (Bhayana & Duckworth, 1984). It is likely then that there are similarities in the structure of the active sites of the two enzymes, a suggestion supported by our conclusions from the proteolytic data that in both cases the dimer is the catalytic unit.

Finally, it is important to realize that the precise positions of the calculated curves in Figs. 1, 3 and 5 are dependent on the assumptions inherent in the derivation of the equations describing them. For example, failure to separate proteolysed and native subunits would displace the curves towards the ordinate, whereas displacement towards the abscissa would be caused by the proteolysed subunits having fractional catalytic activity. Differential susceptibility to proteolysis of forms A to G of the hexameric protein could move the curves in either direction depending on whether proteolysis was increased or decreased.

In conclusion, the data in this paper provide the first direct evidence for the dimer being the functional unit of all types of citrate synthase. This is retained even in the hexameric forms of the enzyme, an apparent advantage of the greater oligomeric association in these being the acquisition of allosteric regulatory sensitivity. Of immediate interest now is to determine precisely how similar the dimer of the large citrate synthases is to that of the small enzymes, and how the association of dimers permits sensitivity to regulatory metabolites. An understanding of how an enzyme's oligomeric nature can determine its functional properties is one of the most interesting problems to which a study of citrate synthases can make an important contribution.

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