Limited Proteolysis of Pig Heart Citrate Synthase by Subtilisin, Chymotrypsin, and Trypsin†

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ABSTRACT: Pig heart citrate synthase was subjected to limited proteolytic attack by subtilisin, chymotrypsin, and trypsin in the presence of palmitoyl-CoA. Initial proteolysis by all three proteolytic enzymes resulted in cleavage of the monomeric subunit (M_r 45 000 ± 3000) into a large (M_r 35 000–38 500) and a small (M_r 9000–12 000) fragment. Further proteolysis of the large subunit produced a secondary fragment (M_r 31 000–36 000). The small (M_r 9000–12 000) fragment was stable in the presence of subtilisin but was substantially degraded by both chymotrypsin and trypsin. The actual molecular weight of fragments varied with the choice of the proteolytic enzyme. Limited proteolysis was absolutely dependent on the presence of palmitoyl-CoA and resulted in complete inhibition of the catalytic activity of the enzyme. Citrate, ammonium sulfate, and especially oxaloacetate provided complete protection against proteolysis whereas acetyl-CoA, CoASH, NADH, and ATP were ineffective. Reaction of rabbit anti-citrate synthase with citrate synthase and its proteolytic fragments indicated that the main antigenic region lay primarily in the small fragment. The products of subtilisin cleavage were isolated by gel filtration under denaturing conditions. The large (M_r 35 000–38 500) fragment contained the amino-terminal (~)336 amino acids and the small fragment contained the remaining carboxyl-terminal amino acids. The results are discussed in relation to the structure of citrate synthase.

Recent studies by Wiegand et al. (1979) on the X-ray crystal structure of pig heart citrate synthase [EC 4.1.3.7; citrate oxaloacetate-lyase (CoA acetylating)] have provided a working model for the three-dimensional structure of the enzyme. The detailed interpretation of this structure would be greatly enhanced by the knowledge of the amino acid sequence of this enzyme. Since the determination of the sequence by conventional cyanogen bromide cleavage at methionine or by total tryptic cleavage would generate very complex mixtures of peptides (see the amino acid composition in Table IV), we felt that the problem might be simplified if large well-defined fragments of citrate synthase could be produced conveniently by limited proteolysis (Linderstrøm-Lang & Ottesen, 1947). Preliminary investigations by Wiegand et al. (1979) showed that the enzyme was susceptible to limited tryptic attack but, under the conditions employed, considerable degradation of fragments was observed. As a main part of our goal we set out to define the exact site of the proteolytic reaction and to isolate the cleavage products. In addition, we show that limited proteolysis of citrate synthase provides important information on the structural features of the enzyme which seem to be consistent with the X-ray analysis of the crystalline protein.

Materials and Methods

Citrate Synthase. The enzyme was prepared from pig hearts following the method of Srere (1969) except that the scale was increased 25-fold. Pure enzyme was obtained after four crystallizations. The majority of the work described in this paper was performed on a single preparation of 350 mg of enzyme; however, the reproducibility of results was confirmed on three other small preparations. Pure citrate synthase had a specific activity at 20 °C of 140–160 units/mg of protein when assayed by monitoring CoASH formation following reaction with 5,5′-dithiobis(2-nitrobenzoate) (Ellman, 1959; Srere, 1969). Protein concentrations were calculated by assuming an A_{280}(0.1%) of 1.78 (Singh et al., 1970).

Antisera to Citrate Synthase. Rabbits were injected on four occasions with 1 mg of citrate synthase in 1 mL of Freund's complete adjuvant. The first three injections were given at 1-week intervals and the fourth was given after a 2-week period. Two weeks after the last injections, rabbits were bled and sera were obtained. The sera were purified further by ammonium sulfate fractionation (Hudson & Hay, 1976).

For quantitative immunoprecipitation, 9 μg of citrate synthase was reacted for 60 min at 0 °C with up to 100 μL of antisera (19 mg of protein/mL) in 0.5 mL of 0.1 M Tris-HCl, pH 8. The precipitate was then collected by centrifugation at 10000*g* for 15 min. The pellet was rinsed with distilled water and dissolved in 200 μL of 0.1 M NaOH for 60 min at 45 °C. The protein was assayed by binding to Coomassie brilliant blue (Bradford, 1976) with bovine serum albumin as a standard. At equivalence, 9 μg (0.1 nmol based on the dimer M_r) of citrate synthase binds 32 μg of antibody (equivalent to 0.2 nmol of IgG with M_r 160000). Thus, there are at least two antigenic sites on each citrate synthase subunit.

Standard Conditions for Limited Proteolysis. Citrate synthase (10 mg/mL) in ammonium sulfate suspension was collected by centrifugation at 10000*g* for 20 min at 0 °C. The enzyme was dissolved in 1 mL of 0.1 M Tris-HCl, pH 8, and dialyzed for 18 h against 1 L of this buffer. After collection, the enzyme was diluted to 5.6 mg/mL with buffer, and an appropriate volume was mixed with an equal volume of 0.5 mM palmitoyl-CoA in water. This gives a mixture containing 2.8 mg/mL citrate synthase (60 μM in subunits), 250 μM palmitoyl-CoA (4 molecules/subunit), and 50 mM Tris-HCl, pH 8.0. The reaction mixture was warmed to 20 °C, and 28 μL of subtilisin, chymotrypsin, or trypsin solutions (1 mg of protein/mL) was added to give a citrate synthase/proteolytic enzyme weight ratio of 100:1. Limited proteolysis was monitored either by loss of catalytic activity or by change in subunit molecular weight upon $NaDodSO_4$ gel electrophoresis. For assays of enzyme activity, 5 μL of enzyme solution was first diluted 200-fold with 0.1 M Tris-HCl, pH 8, to stop proteolysis, and an aliquot was then assayed. Following dilution, there was no further change in enzyme activity over 24 h. For

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estimation of subunit molecular weight, 25 μL of reaction mixture was treated with 5 μL of 10 mM phenylmethanesulfonyl fluoride to inhibit the proteolytic enzyme (Fahrney & Gold, 1963; Polgar & Bender, 1967). After 15 min at 0 °C, 50 μL of 1% (w/v) $NaDodSO_4$ containing 100 mM β-mercaptoethanol and 5 mM sodium phosphate, pH 7, was added and boiled for 2 min. An aliquot (25 μL) was analyzed by $NaDodSO_4$ gel electrophoresis on 10% polyacrylamide gels. The system used was slightly modified from the Weber & Osborn (1969) procedure in that the running buffer was 50 mM sodium phosphate, pH 8.6, containing 0.2% (w/v) $NaDodSO_4$ and the gel buffer was 0.1 M sodium phosphate, pH 7, containing 0.2% (w/v) $NaDodSO_4$. At the end of electrophoresis, gels were stained with Coomassie brilliant blue. Following destaining, the amount of protein in each band was measured by using a Gilford 240 spectrophotometer equipped with a gel scanning device operated at 540 nm.

Nomenclature of Polypeptides. All polypeptides examined in the present work are named according to the system [(CS) (cleavage) ($M_r \times 10^{-3}$)]. The abbreviations used for the nomenclature are CS (citrate synthase), tryp (TPCK–trypsin), chymo (chymotrypsin), and subt (subtilisin-BPN). The molecular weights quoted are subject to an error of ±5%. Calculations of the number of amino acid residues in a polypeptide are tentative and based on an average residue molecular weight of 110.

Purification of CS $subt_{37}$ and CS $subt_{10}$. Citrate synthase (50 mg, ~1 μmol in subunits) in 18.5 mL of 50 mM Tris-HCl, pH 8, containing 250 μM palmitoyl-CoA and 500 μg of subtilisin-BPN was reacted at 20 °C until the enzyme was 90% inhibited (40–60 min). Reaction was terminated by the addition of 50 μL of 75 mM phenylmethanesulfonyl fluoride (Polgar & Bender, 1967). After 15 min at 0 °C, the enzyme was dialyzed exhaustively vs. water or reduced and carboxymethylated. In the latter case 14 g of guanidine hydrochloride and 1 mL of 0.1 M EDTA, pH 8, were added and the pH was adjusted to pH 8.6 with solid Tris. Dithioerythritol (100 mg) was added and reduction was allowed to proceed for 2 h at 30 °C. Iodoacetic acid (268 mg) in 1 mL of 1 M NaOH was added and carboxymethylation was continued for 15 min in the dark at 20 °C. After the addition of 50 mg of dithioerythritol, the sample was dialyzed exhaustively vs. water. Both samples were freeze-dried and dissolved in 4 mL of 6 M guanidine hydrochloride containing 100 mM ammonium bicarbonate. They were purified by chromatography on Sephadex G-75 SF (110 × 2.5 cm) equilibrated with the guanidine solution (flow rate = 5 mL/h). Fractions containing CS $subt_{37}$ and CS $subt_{10}$ were identified by $NaDodSO_4$ electrophoresis, pooled, and dialyzed exhaustively vs. water. Rechromatography of both fractions under the same conditions yielded pure polypeptides.

Analytical Techniques. Automated Edman degradations were performed as described by Koide et al. (1978) for the liquid-phase mode in a Beckman sequencer. The PTH-amino acids were identified by high-pressure liquid chromatography (Hermann et al., 1978). Amino acid analysis was performed with a Durrum D-500 amino acid analyzer.

Results

Subunit Structure. $NaDodSO_4$ gel electrophoresis on 10% acrylamide gels showed that citrate synthase migrated as a single band with a molecular weight of 45 000 ± 3000. This molecular weight is consistent with the proposed structure for the native enzyme as a dimer of molecular weight 100 000 (Wu & Yang, 1970). Assuming a subunit molecular weight of 45 000 and a mean residue molecular weight of 110, there are ~409 ± 27 amino acids in the sequence.

Table I: Amino-Terminal Sequences of Polypeptides Derived from Citrate Synthase

polypeptide	amino-terminal sequence
citrate synthase	Ala-Ser-Ser-Thr-Asn-Leu-Lys-Asp-Ile-Leu-Ala-Asp-Leu-Ile-Pro-
CS $subt_{37}$	Ala-Ser-Ser-Thr-Asn-Leu-Lys-Asp-Ile-Leu-
CS $subt_{10}$	Val-Leu-Lys-X-Asx-Asp-Pro-Tyr-

The amino-terminal sequence of the first 15 amino acids in the citrate synthase polypeptide chain is shown in Table I. The initial sequence Ala-Ser-Ser-Thr was taken as characteristic for the amino-terminus of the protein. The amino-terminal segment was heterogeneous since the first turn of the sequencer showed that both Ala and Ser were present in the ratio 3.6:1. However, it was recognized that this was due to the loss of amino-terminal Ala from 22% of the polypeptide chains.

Inhibition by Proteolytic Enzymes. Previous work by Wiegand et al. (1979) showed that in the presence of palmitoyl-CoA, tryptic cleavage of citrate synthase produced a fragment of ~36 000 M_r. Treatment of citrate synthase with trypsin, chymotrypsin, or subtilisin resulted in a time-dependent inhibition of enzyme activity, and after prolonged reaction periods, the enzyme was completely inhibited. The effectiveness of the proteolytic enzymes (weight/weight ratio enzyme/proteolytic enzyme of 1:100) decreased in the order trypsin > chymotrypsin > subtilisin. No inhibition by proteolytic enzymes was observed in the absence of palmitoyl-CoA. Palmitoyl-CoA is a powerful inhibitor of citrate synthase (Wieland et al., 1964), but inhibition was rapidly reversed by dilution. To assay the enzyme activity, we diluted the reaction mixture containing 250 μM palmitoyl-CoA 5000-fold, giving a palmitoyl-CoA concentration of 50 nM.

Identification of the Main Proteolytic Fragments of Citrate Synthase. Limited proteolysis by subtilisin, chymotrypsin, or trypsin was performed under standard conditions for various times. The products of proteolysis and their molecular weights were identified by $NaDodSO_4$ gel electrophoresis. Although the three proteolytic enzymes showed quite distinct specificities toward synthetic substrates, they produced qualitatively similar proteolytic fragments from citrate synthase. Initially, during short reaction periods, a primary fragment was formed (35 000–38 500 M_r), accompanied by a lower molecular fragment (9000–12 000 M_r). As the reaction continued, the amount of primary fragment decreased and was accompanied by the appearance of a secondary fragment (31 000–36 000 M_r). A detailed kinetic analysis of these results is shown in Figure 1. With all three proteolytic enzymes, the disappearance of native citrate synthase resulted in the primary formation of a high and a low molecular weight fragment. Both fragments were produced without an apparent lag period, showing that they must represent the initial cleavage products. In the case of both subtilisin and trypsin, the sum of the molecular weights of the primary fragments is in close agreement with the observed molecular weight of the native subunit. The action of chymotrypsin was somewhat different in that three small fragments, having molecular weights of 12 000, 9000, and 6000, were observed. The 12 000 M_r fragment was the main small fragment after short incubation periods and probably represents a primary cleavage product (Figure 1B). The 9000 and 6000 M_r polypeptides probably represent intermediates in the breakdown of the 12 000 M_r fragment. The resistance of the small fragment to proteolysis evidently depends upon the proteolytic enzyme. The small

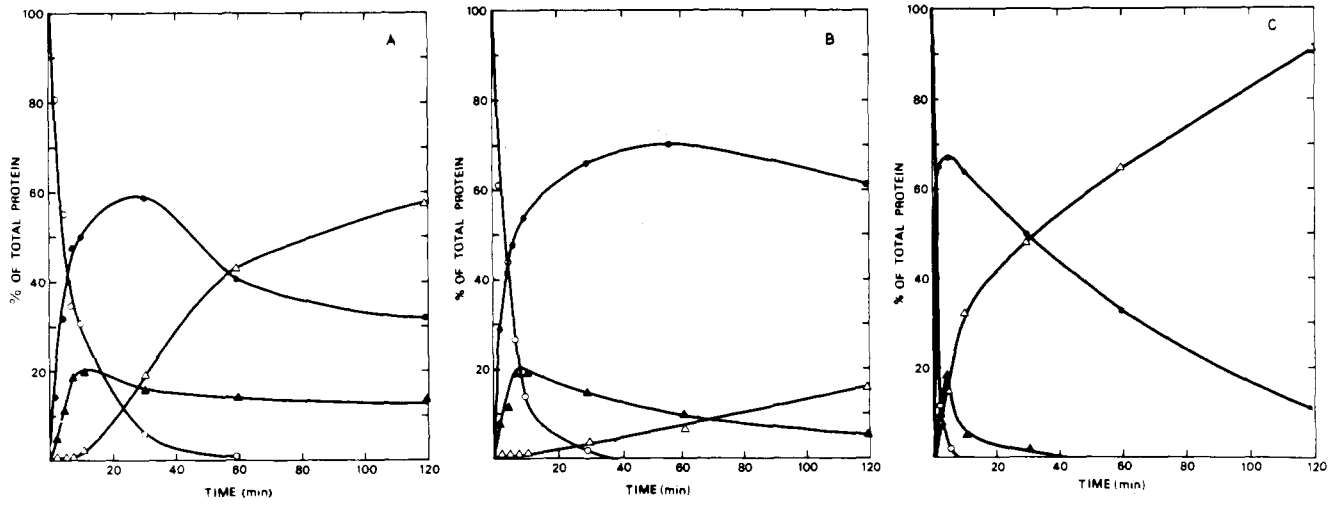

FIGURE 1: Limited proteolysis of citrate synthase by subtilisin (A), chymotrypsin (B), and trypsin (C) under standard conditions for varying times. The amount of native subunit (○), primary fragment (●), secondary fragment (△), and small fragment (▲) was determined after NaDodSO$_4$ gel electrophoresis and staining with Coomassie brilliant blue.

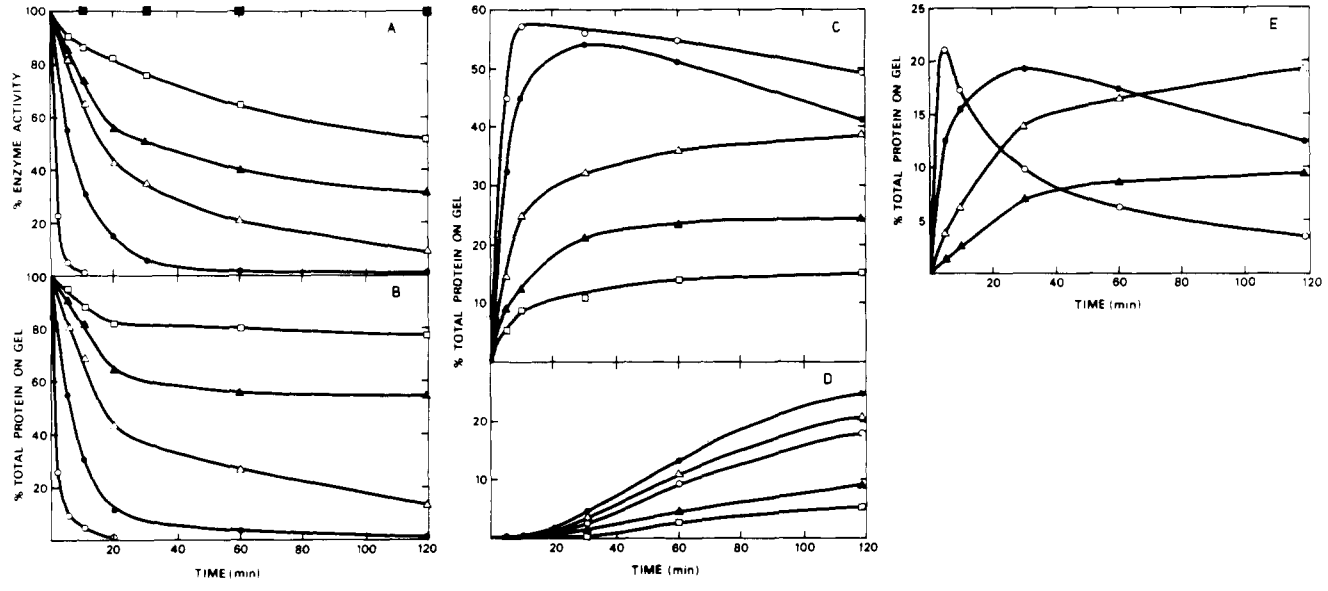

FIGURE 2: Time-dependent proteolysis of citrate synthase (60 μM in subunits) by chymotrypsin in the presence of 500 (○), 240 (●), 120 (△), 60 (▲), 30 (□), and 0 μM (■) palmitoyl-CoA. (A) Enzyme activity; (B) enzyme subunit concentration; (C) primary fragment concentration; (D) secondary fragment concentration; (E) the small fragment (M_r 12 000) concentration.

fragment was relatively stable in the presence of subtilisin (Figure 1A), decomposed slowly via observable intermediates in the presence of chymotrypsin (Figure 1B), and was quite unstable in the presence of trypsin (Figure 1C). This result indicated that the best opportunity for isolation of the primary large and small fragments came from limited proteolysis with subtilisin. Kinetic analysis of the data also revealed that the secondary product was derived by proteolytic action on the primary product. Subtilisin and chymotrypsin treatment showed a clear time lag of the appearance of the secondary product. Also, with all three proteolytic enzymes, the amount of the secondary fragment continued to increase after the native enzyme had disappeared. This could only occur if the primary cleavage product served as the precursor for the secondary one. This was demonstrated for proteolysis by trypsin and subtilisin, which in the latter stages of digestion showed a decline of the primary product and an increase in the secondary product.

Dependence of Proteolysis on Palmitoyl-CoA Concentration. The influence of palmitoyl-CoA on the kinetics of formation of chymotryptic fragments and the inhibition of enzyme activity is shown in Figure 2. The inclusion of 30 μM palmitoyl-CoA (0.5 molecule per monomer) was sufficient to stimulate the proteolytic reactions. With each proteolytic enzyme, a concentration-dependent enhancement of the initial reaction was observed. However, the result was sometimes more complex over longer reaction periods as illustrated by the fact that high palmitoyl-CoA concentrations enhance the degradation of the small fragment (Figure 2E). The rate of formation of the secondary fragment appeared to be saturated at 120 μM palmitoyl-CoA (Figure 2D). Possibly, cleavage at the second site is not strictly palmitoyl-CoA dependent. Thus, after 120 min, the concentration of the secondary fragment was 38 ± 5% of that of the primary fragment and independent of palmitoyl-CoA concentration. If the secondary proteolytic reaction had been palmitoyl-CoA dependent, this value would have increased with increasing palmitoyl-CoA concentration.

Data from parts A and B of Figure 2 were used to construct correlation curves of enzyme inhibition and disappearance of the native subunit (Figure 3). Unexpectedly, palmitoyl-CoA concentrations greater than 120 μM (2 molecules per subunit) gave a slope near unity (0.98) when enzyme inhibition was plotted vs. disappearance of native subunit, showing that the

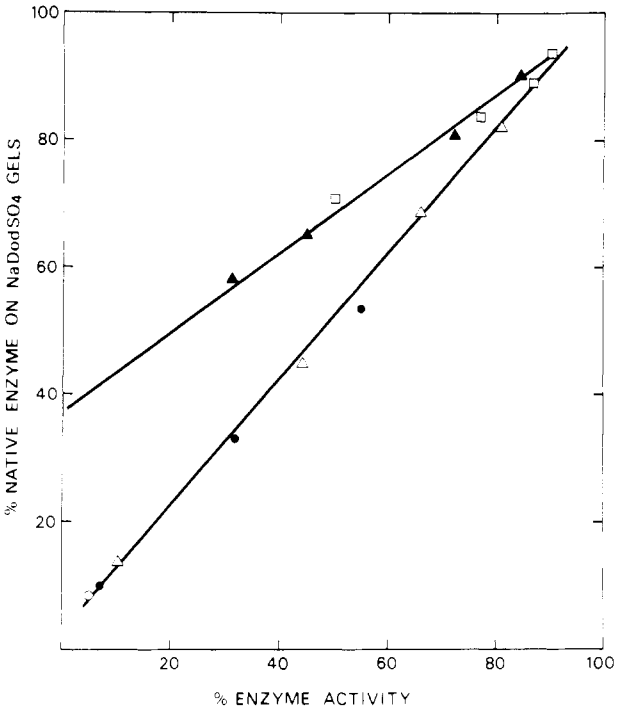

FIGURE 3: Correlation of loss of enzyme activity with loss of native subunit in the presence of 500 (○), 240 (●), 120 (△), 60 (▲), and 30 μM (□) palmitoyl-CoA.

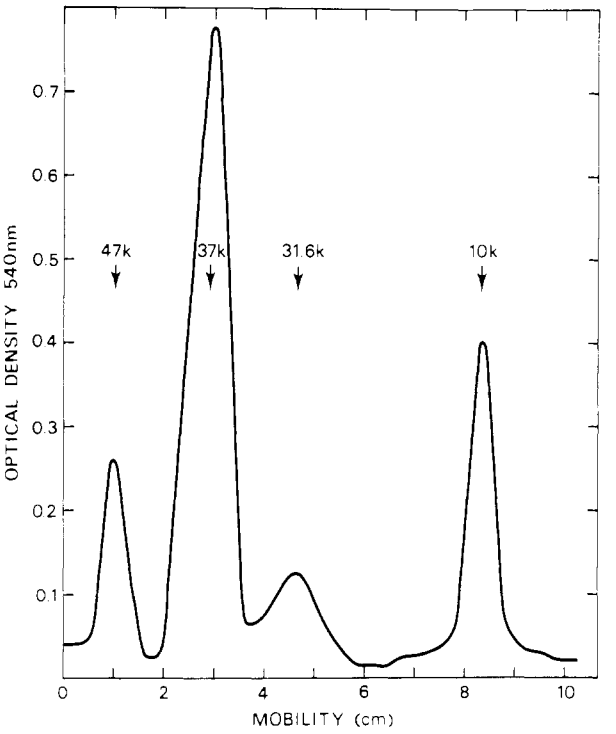

FIGURE 4: Analysis of the products of limited proteolysis of citrate synthase by subtilisin. Citrate synthase was treated with subtilisin until 90% inhibition was reached, and the products were then analyzed by $NaDodSO_4$ gel electrophoresis. The illustration shows a densitometer tracing of Coomassie brilliant blue stained gels.

two parameters were closely related. At lower palmitoyl-CoA concentration, the slope of the line decreased to 0.58. Extrapolation to 0% enzyme activity indicates that, on the average, 40% of the subunits retained the native molecular weight when the enzyme was completely inactivated.

Ligand Protection against Limited Proteolysis. Evidence for ligand protection of citrate synthase against limited proteolysis was initially obtained by testing the effect of both the mode of enzyme preparation and the influence of buffer on the rate of enzyme inhibition by proteolytic enzymes. The basic observations were twofold. First, proteolysis proceeded faster in Tris-HCl buffer than in *N*-methylmorpholine buffer, showing that buffer composition influenced proteolysis. Second, the rate of proteolysis in either buffer was enhanced when the citrate synthase was dialyzed rather than centrifuged, probably due to more efficient removal of ammonium sulfate by dialysis. Increasing ionic strength has a profound effect on citrate synthase activity, and this is especially true for polyvalent anions such as sulfate which is a competitive inhibitor of citrate synthase (Srere et al., 1973). The suggestion that sulfate might reduce the susceptibility to proteolysis prompted us to test a series of ligands including all the substrates and products of the catalytic reaction on proteolysis by chymotrypsin, subtilisin, and trypsin. The ligands tested included oxaloacetate, citrate, acetyl-CoA (0.25–2.0 mM), CoASH (0.5–2.0 mM), and the inhibitors NADH [0.1–5.0 mM, Weitzman (1966)], ATP [0.5–5.0 mM, Shepherd & Garland (1966)], and ammonium sulfate (5–250 mM). Of these, acetyl-CoA, CoASH, ATP, and NADH (i.e., all the nucleotide-related substrates or inhibitors) were completely ineffective in providing protection against proteolysis. ATP provided some protection but only 10–20% at the highest concentration used. However, oxaloacetate, citrate, and ammonium sulfate all completely protected at saturating concentrations. For protection against proteolysis by the three enzymes, the mean $K_{oxaloacetate}$ was estimated at $2.4 \times 10^{-4} \pm 0.7 \times 10^{-4}$ M (range 1.6×10^{-4}–2.8×10^{-4} M). $K_{citrate}$ and $K_{ammonium\ sulfate}$ were estimated as 1.8×10^{-2} M and 6.3×10^{-2} M, respectively. Ligand protection decreased the reaction velocity but did not alter the site of proteolytic cleavage.

Immunoreactivity of Citrate Synthase Cleaved by Limited Proteolysis. We were interested in conformational changes in citrate synthase and in testing whether the small fragment removed by proteolysis was located on the surface of the protein. Valuable information on both these aspects could be found by investigating the interaction of citrate synthase and its proteolytically modified forms with antisera. For this study, proteolytically modified forms of citrate synthase of relatively simple composition were isolated under nondenaturing conditions. One preparation was isolated after 120-min incubation of citrate synthase with trypsin under standard conditions and consisted solely of the secondary fragment, CS $tryp_{36}$ (~372 amino acids). The conditions of isolation lead to complete destruction of the small molecular weight fragment (Figure 1). Another preparation was isolated after incubating citrate synthase with subtilisin until 90% of enzyme activity was lost. This species contains CS $subt_{37}$ (~336 amino acids) and CS $subt_{10}$ (CS $subt_{37+10}$) in the same mixture (Figure 4). If CS $subt_{37}$ and CS $tryp_{36}$ originate from the same region of the protein, then they are virtually the same polypeptide. It follows that the difference between the two proteolytically modified forms of citrate synthase is due to the presence or absence of CS $subt_{10}$ (i.e., the smaller molecular weight fragment). Ouchterlony double diffusion of citrate synthase antisera vs. citrate synthase, CS $tryp_{36}$, and CS $subt_{37+10}$ showed that all three species formed a single precipitin line (Figure 5, top row and center row, left) although that due to CS $tryp_{36}$ was somewhat fainter. When the samples were mixed, citrate synthase and CS $subt_{37+10}$ reacted identically to give a single precipitin line (Figure 5, center row, right) whereas both forms were different from CS $tryp_{36}$ as shown by spurs in the precipitin line (Figure 5, bottom row). These results were evaluated more closely by quantitative immunoprecipitation

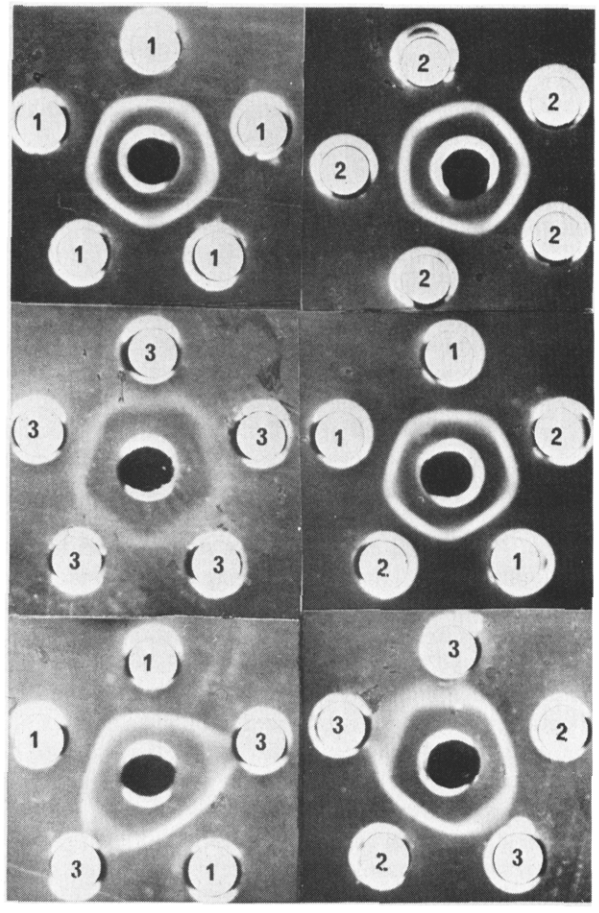

FIGURE 5: Immunodiffusion of rabbit anti-citrate synthase (center well) against citrate synthase and its proteolytic fragments. The additions to each well were (1) citrate synthase, (2) CS $subt_{37+10}$, and (3) CS $tryp_{36}$.

which showed that both citrate synthase and CS $subt_{37+10}$ gave virtually identical amounts of precipitate at equivalence whereas the immunoprecipitate with CS $tryp_{36}$ was substantially reduced to ~20%.

It is tempting to suggest that the main region of immunoreactivity in citrate synthase is located on the low molecular weight fraction. Huber and co-workers (R. Huber, personal communication) have shown that CS $tryp_{36}$ contains the amino-terminal 327 amino acids. We set out to prove that the CS $subt_{37}$ contained the amino terminus of the protein and that CS $subt_{10}$ contained the carboxyl terminus.

Isolation of CS $subt_{37}$ and CS $subt_{10}$. In order to take advantage of the specific subtilisin cleavage for designing a strategy for sequencing citrate synthase, or for other studies of the enzyme, it is important to establish whether CS $subt_{37}$ and CS $subt_{10}$ can be isolated in relatively pure form. To this end, citrate synthase was subjected to subtilisin cleavage under standard conditions until 90% of the enzyme activity was lost. This extent of reaction was judged optimum from kinetic studies (Figure 1) which showed that accumulation of the secondary fragment would not interfere with the isolation of the primary proteolytic products. At the end of the reaction, subtilisin was inhibited by the addition of phenylmethanesulfonyl fluoride (2 mM final concentration), salts were removed by exhaustive dialysis vs. water, and the product was freeze-dried. Alternatively, the products were reduced and carboxymethylated prior to dialysis. Both products were purified by chromatography on Sephadex G-75 SF in 6 M guanidine hydrochloride containing 100 mM ammonium bicarbonate (Figure 6). CS $subt_{37}$ eluted from the column in

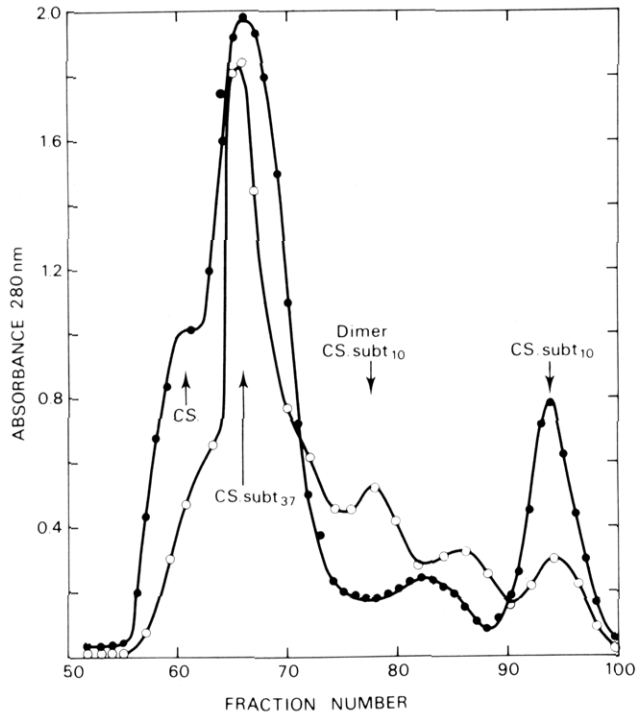

FIGURE 6: Purification of CS $subt_{37}$ and CS $subt_{10}$ on Sephadex G-75 SF in 6 M guanidine hydrochloride containing 100 mM ammonium bicarbonate using either native (○) or reduced and carboxymethylated (●) subtilisin-treated citrate synthase as the starting material.

Table II: Approximate Amino Acid Composition[a] (Residues per Molecule) of Citrate Synthase and Proteolytic Fragments

amino acid[b]	CS $subt_{37}$	CS $subt_{10}$ dimer	CS $subt_{10}$ monomer	citrate[c] synthase	CS $subt_{37}$ plus CS $subt_{10}$
Asp	33	9	9	40	42
Thr	19	5	5	24	24
Ser	23	6	6	29	29
Glu	34	9	9	40	43
Pro	16	6	5	25	21
Gly	35	7	7	35	42
Ala	27	7	7	36	34
Val	19	7	6	29	25
Met	8	1	1	12	9
Ile	17	3	3	15	20
Leu	37	11	11	47	48
Tyr	16	3	4	17	20
Phe	10	3	3	12	13
His	11	3	3	13	14
Lys	19	6	6	23	25
Arg	15	4	4	19	19

[a] Amino acid composition is reported as the values after 24-h acid hydrolysis without correction for selective decomposition of amino acids and hence should be considered tentative. [b] Cysteine was not determined because there was no attempt to stabilize SH bonds during isolation. [c] The amino acid composition of citrate synthase was provided by R. Huber (personal communication).

the volume calibrated for a polypeptide of 37 000 M_r; however, CS $subt_{10}$ eluted both as the monomer and as the dimer. The dimeric form was obtained in the greatest yield under nonreducing conditions whereas the monomer was the major form after reduction and carboxymethylation. Amino acid analysis (Table II) and $NaDodSO_4$ gel electrophoresis (not shown) demonstrated that the monomeric and dimeric fractions were identical. The sum of the amino acid compositions of CS $subt_{37}$ and CS $subt_{10}$ was in reasonable agreement with the amino acid composition of the intact enzyme. This indicates that subtilisin treatment does not result in the loss of a sig-

nificant segment of the molecule which may have escaped isolation by chromatography.

CS $subt_{37}$ contained the amino-terminal sequence Ala-Ser-Ser-Thr (Table I) characteristic of the amino-terminal region of the native protein whereas CS $subt_{10}$ contained a new sequence (Table I). It was clear from the sequenator run that the polypeptide was not homogeneous. For example, the first turn of the sequenator showed the presence of Ala/Val/Leu in the ratio 0.3:1.0:0.45. We interpret this result to indicate the presence of three staggered sequences, Ala-Val-Leu-Lys-X-Asx-Asp-Pro-Tyr-, Val-Leu-Lys-X-Asx-Asp-Pro-Tyr-, and Leu-Lys-X-Asx-Asp-Pro-Tyr-. The stagger was easily recognized in the sequenator run.

Since CS $subt_{37}$ contains the amino-terminal sequence of the native enzyme, we conclude that subtilisin cleaves the enzyme between residue 336 and residue 337 (approximate numbers).

Discussion

Location of Cleavage Point by Subtilisin. The primary site of proteolytic attack by subtilisin has been unambiguously demonstrated to lie in the carboxyl-terminal region of the protein at the approximate position of residues 336 to 337. Parallel studies of tryptic proteolysis (R. Huber, personal communication) show that the general region of hydrolysis is the same but the actual residue is somewhat different (approximate residues 346 to 347). This is seen in the fact that the primary protein product of tryptic proteolysis (CS $tryp_{38}$) is slightly larger than that of subtilisin proteolysis (CS $subt_{37}$). We assume that chymotryptic proteolysis also occurs in the carboxyl-terminal region to yield a primary protein product (CS $chymo_{35}$). If this is true, then it means that although the three enzymes have different specificities for the hydrolysis of peptide bonds, their action on citrate synthase is restricted to a small region stretching across ~29 residues (318–346). The specificities of the enzymes are of course reflected by the slight difference in the actual site of attack; however, the close proximity of the three sites suggests that there could be some unique structural feature in the susceptible region of the protein.

Is there a general theory to account for the action (or more properly the restricted action) of proteolytic enzymes on native enzymes? At the moment there does not seem to be a comprehensive description for this process, but a recent model has been presented by one of us (Neurath, 1980). In this model it is visualized that amino acids in functional domains of proteins are tightly packed and the regions that are most susceptible are those which link domains, and these are referred to as "hinge" regions. Alternatively, it is conceivable that in domains, regions that are solvent exposed may also be susceptible to proteolysis, and these are defined as "fringes". It is interesting to see how the data for the proteolysis of citrate synthase fit into these concepts. The native enzyme is very resistant to limited proteolysis, indicating that it does not contain two distinct domains. However, the addition of palmitoyl-CoA, whether it is acting as a detergent or as a regulatory ligand, results in the specific cleavage of the enzyme into two parts. These two parts may constitute functional domains even if they are not clearly separated in the native protein. Intuitively, we would predict two domains in citrate synthase, one responsible for binding acetyl-CoA or CoA and the other responsible for binding oxaloacetate or citrate. These two domains must cooperate in catalysis as shown by the absolute requirement for *S*-malate in stimulating enzymatic deprotonation of acetyl-CoA (Eggerer, 1965). The observation that only oxaloacetate, citrate, and sulfate provide protection against proteolysis, whereas acetyl-CoA and related ligands do not, suggests that the site of proteolysis could be located in the region of the oxaloacetate domain. Interestingly, acetyl-CoA does not compete at the palmitoyl-CoA binding site, showing that the respective binding sites are quite distinct (Caggiano & Powell, 1979). It seems most probable that the site of proteolysis is a fringe region located in the oxaloacetate domain. Certainly evidence of the immunoreactivity of citrate synthase indicates that the carboxyl-terminal 90 amino acids are located on the surface of the molecule.

Protection by oxaloacetate against proteolysis could occur by one of two mechanisms. First, oxaloacetate may form a binary complex which undergoes a substantial conformational change compared to the native enzyme (Srere, 1966). This might result in decreased proteolytic susceptibility. Second, oxaloacetate may displace palmitoyl-CoA from citrate synthase (Caggiano & Powell, 1979), thus preventing its ability to stimulate proteolysis.

Site of Proteolysis by Subtilisin. Although spatial considerations are important in determining the site of proteolytic action, the residue specificity of subtilisin toward citrate synthase is consistent with observations on synthetic substrates. Model studies by Morihara et al. (1969) show that subtilisin has an extended binding site of approximately five residues, and optimum hydrolysis is observed with leucine or a similar amino acid at the P_1' site [see Schechter & Berger (1967) for details of site nomenclature]. The newly formed amino terminus, equivalent to P_1' in CS $subt_{10}$, is valine or leucine in the ratio 1:0.45. When leucine occupies the P_1' site, then valine is a P_1 which is reasonable for subtilisin specificity. The complete details of P_1, P_2, and P_3 specificity will only be revealed when the carboxyl-terminal sequence of CS $subt_{37}$ is known.

Proteolysis and Citrate Synthase Biosynthesis. The formation of the amino terminus of citrate synthase is also the result of proteolytic processing during biosynthesis. Thus, the anticipated initiator methionine was absent, and the amino terminus was staggered with both Ala and Ser present in a ratio of 3.6:1, indicating proteolytic action. Since it is the carboxyl-terminal region and not the amino-terminal one of native citrate synthase which is subject to limited proteolysis, we may conclude that proteolytic processing in the biosynthesis of the enzyme must occur prior to the acquisition of the native structure.

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Glycolate Formation Catalyzed by Spinach Leaf Transketolase Utilizing the Superoxide Radical†

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ABSTRACT: A homogeneous preparation of transketolase was obtained from spinach leaf; the specific enzyme activity was 9.5 μmol of glyceraldehyde-3-P formed (mg of protein)$^{-1}$ min^{-1}, when xylulose-5-P and ribose-5-P were used as the donor and acceptor, respectively, of the ketol residue. Transketolase catalyzed the formation of glycolate from fructose-6-P coupled with the O_2^--generating system of xanthine–xanthine oxidase. The addition of superoxide dismutase (145 units) or 1,2-dihydroxybenzene-3,5-disulfonic acid (Tiron) (5 mM), both O_2^- scavengers, to the reaction system inhibited glycolate formation 72 and 58%, respectively. The reaction was not inhibited by catalase. Mannitol, an ·OH scavenger, and β-carotene and 1,4-diazobicyclo[2.2.2]octane, $^1O_2$ scavengers, showed little or no inhibitory effects. The rate of glycolate formation catalyzed by the transketolase system was measured in a coupled reaction with a continuous supply of KO_2 dissolved in dimethyl sulfoxide, used as an O_2^--generating system. The optimum pH of the reaction was above pH 8.5. The second-order rate constant for the reaction between transketolase and O_2^-, determined by the competition for O_2^- between nitroblue tetrazolium (NBT) and transketolase, was 1.0×10^6 M^{-1} s^{-1}. Transketolase showed an inhibitory effect on the O_2^--dependent reduction of NBT only if the reaction mixture was previously incubated with ketol donors such as fructose-6-P, xylulose-5-P, or glycolaldehyde. The results suggest the possibility that transketolase catalyzes O_2^--dependent glycolate formation under increased steady-state levels of O_2^- in the chloroplast stroma.

It has been well established that chloroplasts of green plants (Mehler, 1951; Asada et al., 1973, 1974a; Asada & Nakano, 1978) and chromatophores from photosynthetic bacteria (Asami & Akazawa, 1977) can reduce molecular oxygen and produce O_2^-. Rates of O_2^- production as high as 10–20 μmol (mg of Chl)$^{-1}$ h^{-1} have been reported[1] (Radmer & Kok, 1976; Radmer et al., 1978; Asami & Akazawa, 1977; Asada & Nakano, 1978). Much higher rates of O_2 reduction [300 μmol (mg of Chl)$^{-1}$ h^{-1}] have been observed with subchloroplast particles (Lien & San Pietro, 1979). However, O_2^- and other active oxygen derivatives such as ·OH, $^1O_2$, and H_2O_2 are known to be toxic to biological systems. It has often been affirmed that superoxide dismutase, which is ubiquitously present in living organisms, scavenges O_2^- and makes its steady-state concentration low, thus constituting an O_2^--detoxifying device (Fridovich, 1975).

Several investigators have reported the damaging photooxidative effect of oxygen on photosynthetic electron transport systems (Avron, 1960; Forti & Jagendorf, 1960; Jones & Kok, 1966), and Asami & Akazawa (1978) have demonstrated that the photooxidative damage to photophosphorylation in *Chromatium vinosum* under high light intensity and an O_2-containing atmosphere appeared to be caused by O_2^-. Radmer & Kok (1976) have shown that O_2 and NADP can be reduced competitively by photosystem I of chloroplasts. Thus, it is conceivable that the rate of O_2 reduction increases under conditions of low CO_2 pressure and high light intensity, which are the environmental conditions believed to be favorable to photorespiration. The mechanism of O_2 reduction needs to be reexamined in relation to glycolate formation, the key metabolic reaction in photorespiration.

Transketolase (EC 2.2.1.1) has long been thought to be involved in photorespiratory glycolate synthesis, since the enzyme catalyzes the formation of glycolate in the presence of artificial oxidants such as ferricyanide and *p*-benzoquinone (Weissbach & Horecker, 1955; Bradbeer & Racker, 1961) (cf. Figure 1).

Shain & Gibbs (1971) theorized that H_2O_2 generated in photosystem I oxidizes the transketolase–glycolaldehyde addition product. Our previous investigation (Asami & Akazawa, 1977) has shown that transketolase catalyzes O_2^--dependent glycolate formation in the presence of fructose-6-P, and we postulated that this subsidiary reaction of transketolase may have a functional role in scavenging O_2^- in the chloroplast stroma. As an extention of this study, we have further explored the mechanism of glycolate formation by the O_2^--requiring

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[1] Abbreviations used: Chl, chlorophyll; crown ether, dicyclohexyl-18-crown-6; DABCO, 1,4-diazobicyclo[2.2.2]octane; NBT, nitroblue tetrazolium; RuBP, ribulose 1,5-bisphosphate; Tiron, 1,2-dihydroxybenzene-3,5-disulfonic acid; TPP, thiamine pyrophosphate.

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