Evidence for Structural Homology between the Subunits from Allosteric and Nonallosteric Citrate Synthase†

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ABSTRACT: The allosteric, NADH-sensitive citrate synthase of *Escherichia coli* has been submitted to limited proteolysis by subtilisin BPN′. The intact subunits, of 47 000 g/mol, are rapidly converted, with at least 90% activity loss, to fragments of about 32 000 (32K) and 13 500 (13.5K) g/mol; at later times, a smaller fragment of about 7500 g/mol (7.5K) appears, probably by further digestion of the 13.5K polypeptide. Binding of NADH is not affected by this proteolysis, and the fragments remain aggregated as a high molecular weight species. Automatic Edman degradation of the separated products shows that the 32K polypeptide represents the N-terminal two-thirds of the subunit. The 13.5K polypeptide fraction contains three N-terminal sequences: the major one arises from subtilisin cleavage of an Arg–Val bond, and the other two arise from cleavages three and five residues to the N-terminal side of this. The major cleavage site and the surrounding sequence are recognizably homologous to the major subtilisin target in the nonallosteric pig heart citrate synthase [Bloxham, D. P., Parmelee, D. C., Kumar, S., Wade, R. D., Ericsson, L. H., Neurath, H., Walsh, K. A., & Titani, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5381; Bloxham, D. P., Parmelee, D. C., Kumar, S., Walsh, K. A., & Titani, K. (1982) *Biochemistry 21*, 2028]. The 7.5K polypeptide contains two N-terminal sequences, which arise from cleavage on either side of a lysine residue; the sequence of the 17 residues on the C-terminal side of this are partially homologous to residues 370–386 in the pig heart citrate synthase sequence. The finding that subtilisin attacks native *E. coli* and pig heart citrate synthases in the same way suggests that some features of the folding, as well as the sequences of the subunits, may be similar; it raises the possibility that the *E. coli* enzyme owes its allosteric properties to the details of subunit interactions, rather than to an intrinsically different kind of subunit than the nonallosteric pig heart enzyme.

The citrate synthases of Gram-negative bacteria differ from those of Gram-positive bacteria and eukaryotes in that they are subject to allosteric inhibition by NADH (Weitzman, 1966; Weitzman & Danson, 1976). NADH-sensitive citrate synthases are large molecules, consisting in well studied cases of four to six copies of a single size of subunit, of molecular weight of 45 000–50 000 (Weitzman & Danson, 1976; Tong & Duckworth, 1975; Higa et al., 1978; Morse & Duckworth, 1980). Pig heart citrate synthase, the best studied eukaryotic example, has two identical subunits of 48 969 g/mol (Bloxham et al., 1981, 1982); its three-dimensional structure is known (Wiegand et al., 1979; Remington et al., 1982). A variety of other eukaryotic and Gram-positive citrate synthases have been partially characterized [e.g., Higa & Cazzulo (1976), Porter & Wright (1977), Koeller & Kindl (1977), Juan et al. (1977), Harmey & Neupert (1979), and Alam et al. (1982)], and all seem to share the dimeric subunit structure of pig heart citrate synthase.

An interesting possibility is that NADH-sensitive and NADH-insensitive citrate synthases consist of similar kinds of subunits, whose molecular weights and tertiary structures are basically the same. Additional subunit interactions present in the larger NADH-sensitive enzymes, however, would permit the existence of a T or inactive conformational state [the terminology of Monod et al. (1965) is used], thus allowing NADH inhibition to occur. To test this possibility, we have investigated the effect of partial proteolysis on the citrate synthase of *Escherichia coli*, a Gram-negative enzyme that is extremely sensitive to NADH (Weitzman, 1966; Duckworth & Tong, 1976). Wiegand et al. (1979) and also Bloxham et al. (1980) have shown that pig heart citrate synthase is cleaved by trypsin, subtilisin, and other proteases, under mild conditions, into two fragments of molecular weights about 37 000 and 10 000–12 000. Enzyme activity is lost as a result of this treatment [see also Bayer et al. (1981)]. The main site of attack by subtilisin is the Ala–Val bond at positions 321–322 (Bloxham et al., 1981), though at least one other cleavage occurs nearby (Bloxham et al., 1980). We now find that limited proteolysis of *E. coli* citrate synthase gives essentially identical results and that the main site of subtilisin cleavage of this enzyme is recognizably homologous with the corresponding site in pig heart citrate synthase.

Materials and Methods

Enzymes and Reagents. Pure *E. coli* citrate synthase was prepared as previously described (Tong & Duckworth, 1975) or from strain HB101/pHS*gltA*, which harbors a multicopy plasmid containing the complete citrate synthase gene, as described elsewhere (Duckworth & Bell, 1982). Methods for assaying enzyme activity and measuring protein concentration have been reported (Tong & Duckworth, 1975). Subtilisin BPN′, TPCK-trypsin,[1] soybean trypsin inhibitor, chymotrypsin, and thermolysin were from Sigma. Sources of most other chemicals have been given previously (Tong & Duckworth, 1975) or were reagent grade.

Proteolytic Inactivation of Citrate Synthase. All experiments were performed in 20 mM Tris-HCl buffer, pH 7.8,

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[1] Abbreviations: DTT, dithiothreitol; Gdn-HCl, guanidine hydrochloride; PMSF, phenylmethanesulfonyl fluoride; PTH, phenylthiohydantoin; $NaDodSO_4$, sodium dodecyl sulfate; TPCK-trypsin, trypsin pretreated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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containing 1 mM EDTA; this will be referred to as Tris–EDTA buffer. For the thermolysin experiments, 2 mM $CaCl_2$ was added to this. Digestions were at 21 °C except in the case of thermolysin, where 60 °C was used. Activity of citrate synthase was monitored by removing samples, diluting them 200–2000-fold in Tris–EDTA buffer to reduce the digestion rate to a negligible value, and assaying them as soon as possible. For NaDodSO$_4$–polyacrylamide gel electrophoresis, samples were quenched with 2 mM PMSF (final concentration) and then mixed with an equal volume of glacial acetic acid and evaporated to dryness in an evacuated desiccator over solid sodium hydroxide. The residue was dissolved in NaDodSO$_4$ sample buffer (see below) by heating 3 min in boiling water.

Isolation of Subtilisin Cleavage Products under Denaturing Conditions. Citrate synthase, 50 mg, was digested with 250 μg of subtilisin BPN′ in a final volume of 15 mL of Tris–EDTA buffer until 85% inactivation was achieved. Digestion was then quenched by the addition of PMSF (Sigma), 50 μL of a 13 mg/mL solution in acetone, and the mixture was cooled in an ice–water bath. After 15 min, 16 g of solid Gdn-HCl, 100 mg of DTT, and 1 mL of 0.1 M EDTA were added, the pH was adjusted to 8.6 with solid Tris base, and the mixture was incubated 2 h at 30 °C to reduce thiol groups. Iodoacetic acid, 286 mg in 1 mL 1 M NaOH containing 50 μCi of iodo[2-^{14}C]acetic acid, was then added, and the mixture was kept at 21 °C in the dark for 15 min. Then, 50 mg of DTT was added, and the mixture was placed in Spectrapor 3 membrane tubing (Fisher), dialyzed exhaustively against deionized water, and lyophilized. The residue was dissolved in 3 mL of 6 M Gdn-HCl–0.1 M NH_4HCO_3 and loaded onto a Sephadex G-75 column, 2.5 × 125 cm, in the same solution. Elution of the column with this solution gave fractions that were monitored for A_{280} and radioactivity; groups of two to four fractions were pooled, dialyzed in Spectrapor 3 membrane tubing against water, lyophilized, and examined further by NaDodSO$_4$–polyacrylamide gel electrophoresis and amino acid analysis.

Isolation of Two Small Arginine Peptides from Citrate Synthase. These peptides were isolated as part of an extensive sequencing project on citrate synthase, which will be described elsewhere. Briefly, 171 mg of reduced, carboxymethylated citrate synthase was subjected to citraconylation by the method of Atassi & Habeeb (1972) as modified by Weng et al. (1978), dialyzed, and lyophilized. The residue was digested thoroughly with TPCK-trypsin, digestion was terminated with soybean trypsin inhibitor, and the digest was fractionated on Sephadex G-50 in 0.1 M NH_4HCO_3. The peak containing the smallest peptides, including both of those required for this study, was fractionated further on Dowex 50-X2 according to Schroeder (1972). Peptide 1 was found in one peak from the Dowex 50-X2 column and was sequenced after further purification by paper electrophoresis at pH 6.5. Peptide 2 was part of a mixture eluted from the Dowex 50-X2 column as a single peak that was resolved by chromatography on Dowex 1-X2, again according to Schroeder (1972), followed by Bio-Gel P2 gel filtration.

Amino-Terminal Sequencing. Automatic Edman degradations were performed with a Beckman 890C sequencer. For degradation of proteins and products of limited subtilisin digestion, a standard 1 M Quadrol program (Beckman Catalog No. 122974) was used, and for the small peptides, a 0.1 M Quadrol program (Beckman Catalog No. 030176). Sequencer reagents were obtained from Pierce; ethyl acetate contained 0.1% acetic acid, and DTT was added at 15 mg/L to the butyl chloride. Samples were added to the cup as solutions in NH_4HCO_3–NH_4OH or water, and aliquots of these solutions were hydrolyzed and analyzed for amino acids to determine the amounts subjected to Edman degradation. For small peptides, 4 mg of Polybrene (Pierce) was added to the cup as well (Tarr et al., 1978). Recoveries of thiazolinones were corrected from recovery of known amounts of PTH-norleucine, added to each tube of the sequencer fraction collector. Thiazolinones were identified by hydrolysis to the free amino acids [6 M HCl–0.1% $SnCl_2$, 150 °C, 4 h (Mendez & Lai, 1975)] followed by amino acid analysis and by conversion to the PTH-amino acids (1.0 M HCl, 80 °C, 10 min) followed by liquid chromatography on a C_{18} reversed-phase column. For some work an Altex liquid chromatograph was used, equipped with an Ultrasphere-ODS column; elution was according to Somack (1980). More recently, we have used a Perkin-Elmer System 4 apparatus, equipped with an HS-3/C18 column and LC-85 spectrophotometric detector; elution was with a step gradient recommended by Perkin-Elmer (Skiados, 1981).

Purification of Subtilisin-Cleaved Citrate Synthase under Nondenaturing Conditions. Citrate synthase, 50 mg in 1 mL of Tris–EDTA buffer, was digested with 500 μg of subtilisin BPN′ until 90% inactivation was achieved. Digestion was then stopped by the addition of PMSF (Sigma), 50 μL of a 13 mg/mL solution in acetone, and the chilled mixture was loaded directly on a Sephadex G-200 column, 0.9 × 200 cm, prepared in Tris–EDTA buffer containing 50 mM KCl. The column was eluted with the same buffer at 4 °C, and fractions were assayed for A_{280}, for citrate synthase activity, and for ability to bind NADH. For NADH binding measurements, the fluorescence enhancement method was used (Duckworth & Tong, 1976), aliquots of protein being added to 20 μM NADH in Tris–EDTA to give a known protein concentration in the fluorescence cuvette close to 0.11 mg/mL.

Other Methods. Amino acid analysis was according to the general procedures of Moore & Stein (1963), using a single-column method with a Technicon NC-2P analyzer equipped with a Micromeritics Model 725 autoinjector and a Spectra-Physics System I computing integrator. NaDodSO$_4$–polyacrylamide gel electrophoresis was according to Laemmli (1970), on 10–15% slab or tube gels. Samples were prepared for electrophoresis by dissolving dry samples in the NaDodSO$_4$ sample buffer of Laemmli (1970), at a final concentration of 1–2 mg/mL, and incubating in a boiling water bath for 2–3 min. For scanning of tube gels, a Perkin-Elmer Model 555 UV–vis spectrophotometer, equipped with a linear transport accessory, was used.

Results

Partial Proteolysis of E. coli Citrate Synthase. Subtilisin BPN′ inactivates *E. coli* citrate synthase rapidly, at low weight ratios, to the extent of 90% or more. Figure 1 shows the course of inactivation of citrate synthase at 4.8 mg/mL by subtilisin at 24 μg/mL, in Tris–EDTA buffer at 21 °C. In this experiment, the half-time for inactivation was about 12 min. This inactivation was accompanied by the disappearance of citrate synthase subunits (47 000 g/mol on NaDodSO$_4$ gels, 47K) and by the appearance of a prominent new band of mobility corresponding to 32 000 ± 1000 g/mol (32K), plus a weaker band at 13 500 ± 1500 (13.5K) g/mol (Figure 1B). The 13.5K band reached a maximum concentration at between 20 and 40 min, at which time a new band with an apparent molecular weight of 7500 ± 1000 (7.5K) appeared. A very weak band at 24 000 ± 1000 (24K) g/mol also began to appear at the longest times, while the 32K fragment seemed to be

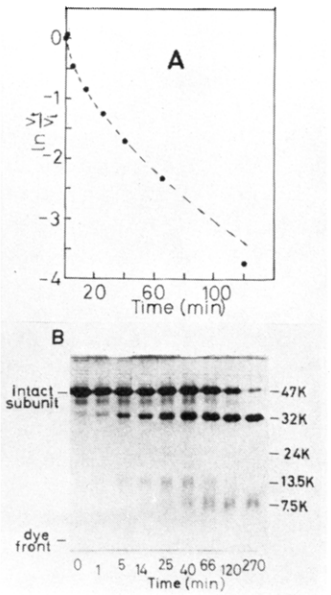

FIGURE 1: Kinetics of loss of *E. coli* citrate synthase activity and production of proteolytic fragments during treatment with subtilisin. Citrate synthase (4.78 mg/mL) in 20 mM Tris-HCl, pH 7.8, containing 1 mM EDTA was incubated with subtilisin BPN′ (24.1 μg/mL) at 21 °C. (A) First-order plot: activity at time t is v_t and activity at "zero time" is v_i. (B) $NaDodSO_4$ gel electrophoresis, on 15% slab gels, of samples (20 μg) taken at times indicated. The zero-time sample was not treated with subtilisin.

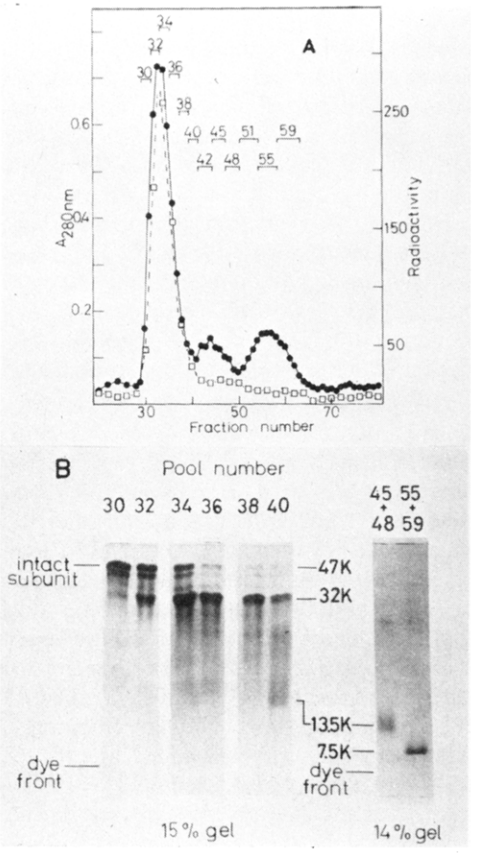

FIGURE 2: (A) Gel filtration of subtilisin cleavage products under denaturing conditions (see Materials and Methods). The Sephadex G-75 column, at room temperature, was developed at a flow rate of about 12 mL/h, and fractions of about 6 mL were collected. A_{280} (●) and radioactivity in dpm/μL (□) were determined. Fractions were pooled as indicated, and the first fraction number was used to designate the pool. (B) Slab gel electrophoresis, on 15% $NaDodSO_4$, gels, of samples (20 μg) taken from the dialyzed and lyophilized pools indicated and on 14% $NaDodSO_4$ gels of samples of pools 45 plus 48 (about 57 μg) and of pools 55 plus 59 (about 43 μg), aliquots of samples sequenced in parts B and C of Figure 3, respectively. The 39.5K contaminant appears as a minor component in pools 30, 32, and 34 with mobility slightly greater than that of the 47K polypeptide. Only trace amounts of the 24K polypeptide can be seen in pools 38 and 40.

relatively stable. The most prominent contaminating band in our citrate synthase preparations, at 39 500 ± 1500 g/mol, disappeared during digestion (Figure 1B).

The rate of inactivation of citrate synthase by subtilisin was decreased by 50% in the presence of 0.1 M KCl, a known activator of the synthase, and it was increased 60% by the inhibitor NADH and 30% by the substrate acetyl coenzyme A, each tested at 0.50 mM. Palmitoyl coenzyme A, which is necessary to obtain subtilisin digestion of undenatured pig heart citrate synthase (Bloxham et al., 1980), had no effect on the digestion of *E. coli* enzyme when tested at 0.50 mM.

Trypsin, chymotrypsin, and thermolysin, each tested at 26 μg/mL, all inactivated *E. coli* citrate synthase (2.6 mg/mL) substantially in 1 h under standard conditions (see Materials and Methods), and in all cases, a large fragment and a small fragment were formed. The exact sizes differed somewhat, depending on the protease used. Except for the fact that palmitoyl coenzyme A was not needed in these digestions, these findings are very similar to those of Bloxham et al. (1980) with the pig heart enzyme.

In experiments to determine the relative yields of the fragments from digestions with the different proteolytic enzymes, we scanned Coomassie Brilliant Blue stained $NaDodSO_4$ rod gels and integrated the areas under the peaks. These measurements showed that the smaller pieces generated by subtilisin digestion were more stable to further breakdown than were those produced by trypsin, chymotrypsin, or thermolysin (data not shown). Accordingly, we chose to study the subtilisin digestion in more detail.

Large-Scale Preparation of Subtilisin Fragments. About 50 mg of *E. coli* citrate synthase was digested with subtilisin to about 85% inactivation, and the product was denatured, reduced and carboxymethylated, and passed through a Sephadex G-75 column in 6 M Gdn-HCl. The elution profile is shown in Figure 2A, and $NaDodSO_4$ gel patterns for pools of selected fractions are given in Figure 2B. The large front peak was found to contain the 47K and 32K polypeptides and the 39.5K contaminant. The pool of fractions 30 and 31 was a mixture of 47K protein and 39.5K contaminant, while the pools of fractions 36 and 37 and 38 and 39 were mainly 32K fragment. The small peak, fractions 42–49, contained mostly 13.5K fragment, with 32K fragment trailing into it. The third peak, fractions 51–65, contained rather pure 7.5K fragment. A small amount of the 24K fragment appeared to be present in fractions 38–41. After dialysis and lyophilization, samples from this profile were submitted to amino acid analysis and N-terminal sequencing.

All samples from the front peak gave a single sequence, which was identical to the N-terminal sequence of intact *E. coli* citrate synthase, published elsewhere but shown in Figure 3A for completeness (Duckworth & Bell, 1982). The pool of fractions 30 and 31 gave an initial coupling of 61%, assuming an equivalent weight of 47 000; that of fractions 36 and 37 gave 73%, assuming the equivalent weight of the 32K fragment. Since little intact citrate synthase subunit (47K) was present in fractions 36 and 37 (Figure 2B), it is clear that the 32K fragment has the same N-terminal sequence as the intact subunit. The stable large fragment from subtilisin cleavage of *E. coli* citrate synthase is shown by these findings to be approximately the N-terminal two-thirds of the subunit, as was found by Bloxham et al. (1980) for the pig heart enzyme.

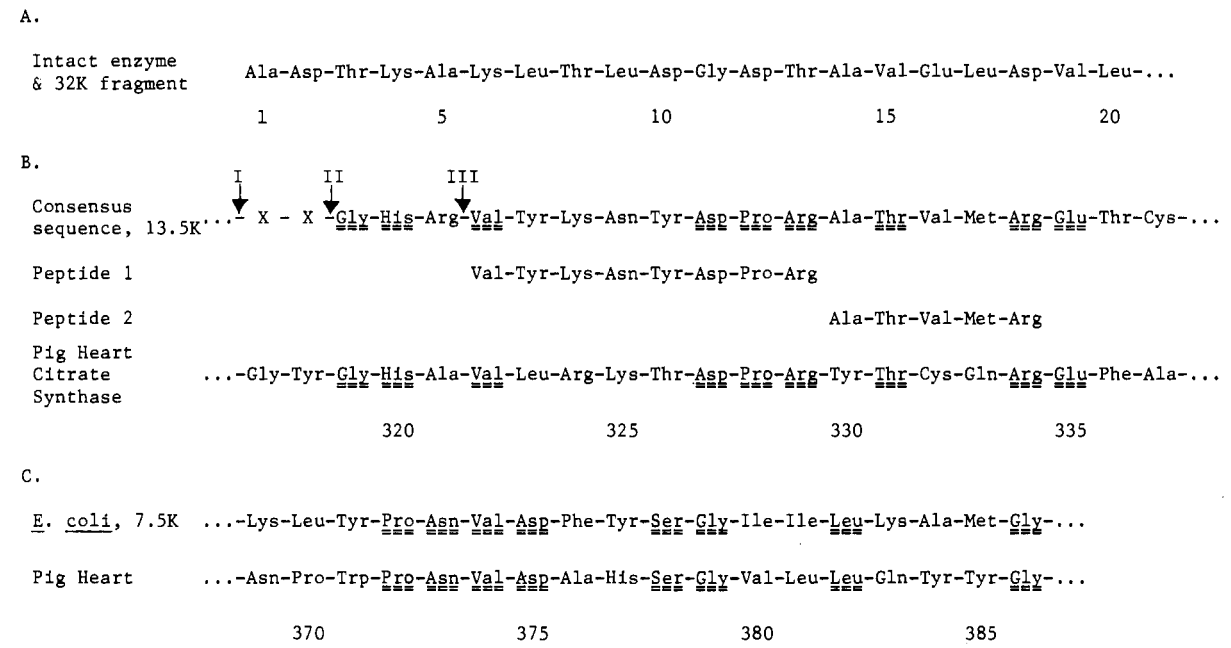

FIGURE 3: N-Terminal amino acid sequences of proteolytic fragments of *E. coli* citrate synthase. (A) Sequence of intact enzyme, taken from Duckworth & Bell (1982). The 32K polypeptide from fractions 36 and 37 (Figure 2) also showed this sequence. (B) Consensus sequence of 13.5K polypeptide from pools 45 plus 48 (Figure 2). Subtilisin cleavage sites are labeled I, II, and III (see text). Peptides 1 and 2 are the sequences of two citraconylated tryptic peptides purified from a total digest of *E. coli* citrate synthase (see Materials and Methods). Pig heart citrate synthease, sequence and numbering in the region of the subtilisin cleavage site between residues 321 and 322, are from Bloxham et al. (1981). Double-underscored residues are the same in the *E. coli* and pig heart sequences. (C) Consensus sequence of 7.5K polypeptide from pools 55 plus 59 (Figure 2). Pig heart citrate synthease sequence and numbering are from Bloxham et al. (1981), for a region homologous to the *E. coli* sequence. Double-underscoring is as in part B.

The second peak in Figure 2A contained several sequences, one of which was easily recognized as the N-terminal sequence of intact citrate synthase. This sequence, representing about 27% of the polypeptide chains in this fraction, cannot be attributed entirely to the small amount of contaminating 32K fragment seen in the $NaDodSO_4$ gel (Figure 2B) and must arise in part from shorter pieces, derived from the N terminus of the subunit, which were not resolved from the 13.5K fragment on the column or the gel. Once the contribution of this sequence had been taken into account, the remaining data could be rationalized by assuming that the 13.5K fragment had arisen by subtilisin cleavage at three neighboring sites: a major one yielding N-terminal valine and two others located three and five residues farther toward the N terminus from this. The consensus sequence for the region adjacent to the subtilisin cleavage sites is shown in Figure 3B, where the three sites are designated I, II, and III. The relative yields of cleavage at these sites, from the sequenator data, were in the ratio 1:1.5:6.5 (I:II:III), and the total initial coupling for the three, assuming an equivalent weight of 13 500, was 57%.

Data of this kind are sometimes subject to ambiguities of interpretation [cf. Bloxham et al. (1981)], but we have confirmed the correctness of a large part of the sequence deduced by isolating two short peptides, derived from this region, from a total tryptic digest of citraconylated citrate synthase. The sequences of these two peptides are also shown in Figure 3B. The sequence obtained for this region shows significant homology with the region of the pig heart citrate synthase sequence near its major subtilisin cleavage site (Bloxham et al., 1981). An alignment of this region with our sequence is also shown in Figure 3B.

The third peak from the Sephadex G-75 column in Figure 2A gave two major sequences, which could be rationalized by assuming that subtilisin had cleaved at two adjacent positions, on either side of a lysine residue, to generate two 7.5K fragments. The yields of the two sequences were in the ratio 1.53:1 (7.5K with N-terminal lysine:7.5K minus lysine). The total initial coupling represented by these two sequences, assuming an equivalent weight of 7500, was 55%. The sequence at the N terminus of the longer 7.5K fragment is shown in Figure 3C, aligned with an apparently homologous region in the sequence of the pig heart enzyme.

The amino acid compositions of the 32K, 13.5K, and 7.5K fragments are shown in Table I, where it is also shown that the compositions of the 32K and 13.5K pieces add up to the

Table I: Amino Acid Compositions of Subtilisin-Generated Major Fragments of *E. coli* Citrate Synthase

amino acid	proteolytic fragments[a] 32K	13.5K	7.5K	sum of 32K plus 13.5K	native enzyme[b]
aspartic acid	33.2	12.6	5.8	45.8	44.3
threonine[c]	17.9	8.0	4.8	25.9	27.5
serine[c]	11.7	6.1	5.4	17.8	21.9
glutamic acid	25.0	9.5	4.1	34.5	31.6
proline	13.2	5.0	3.7	18.2	19.6
glycine	23.9	8.3	5.3	32.2	28.9
alanine	28.7	9.3	4.7	38.0	35.2
valine[d]	13.0	7.0	3.4	20.0	20.1
methionine	8.8	5.4	3.6	14.2	15.9
isoleucine[d]	18.3	8.6	5.5	26.9	27.1
leucine	26.9	9.6	3.7	36.5	36.1
tyrosine	8.7	5.0	3.0	13.7	16.1
phenylalanine	14.9	5.4	3.4	20.3	21.1
histidine	11.0	3.3	1.8	14.3	15.5
lysine	13.3	7.9	4.3	21.2	23.0
arginine	16.2	7.4	4.2	23.6	24.0
cysteine[e]	4.8	1.6	0.6	6.4	7.4
tryptophan	ND[f]	ND	ND	ND	3.2

[a] Amino acid composition of the sample sequenced. [b] From Duckworth & Bell (1982). [c] By first-order extrapolation to zero hydrolysis time. [d] Values from 72-h hydrolysis adopted. [e] Cysteine as CM-cysteine in amino acid analysis. [f] ND, not determined.

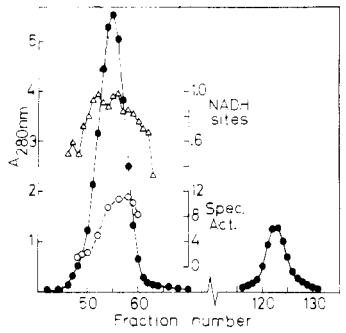

FIGURE 4: Gel filtration of subtilisin-cleaved citrate synthase under nondenaturing conditions (see Materials and Methods). The Sephadex G-200 column, at 4 °C, was developed at a flow rate of about 12 mL/h, and fractions of about 1 mL collected. Shown are A_{280} (●), citrate synthase specific activity in units/mg (○), and ability to bind NADH in mol/mol of subunit (△).

known composition of the 47K subunit previously published (Duckworth & Bell, 1982).

Properties of Undenatured E. coli Citrate Synthase after Partial Proteolysis. A sample of subtilisin-cleaved citrate synthase was passed through a Sephadex G-200 column without denaturation, as shown in Figure 4, and fractions were monitored for A_{280}, citrate synthase activity, and ability to bind NADH. Selected fractions were also examined by NaDodSO$_4$–polyacrylamide gel electrophoresis. The major column peak contained the bulk of the citrate synthase protein. The leading edge was mainly 32K polypeptide, while the trailing edge was mainly uncleaved subunits, coinciding with the small amount of remaining citrate synthase activity (Figure 4). The other major digestion products already described, 13.5K and 7.5K, showed the same distribution across the peak as the 32K polypeptide (data not shown). The small A_{280} peak of low molecular weight material contained no polypeptide large enough to be seen on the Coomassie Brilliant Blue stained NaDodSO$_4$ gels. After acid hydrolysis it was found to contain amino acids, and it probably consists of short peptides released by subtilisin.

The specific binding of NADH per A_{280} unit is constant across the large Sephadex G-200 peak (Figure 4), although the specific enzyme activity changes from less than 1 unit/mg at the front to about 10 units/mg at the back of this peak (the specific activity of pure *E. coli* citrate synthase is 64 units/mg; Duckworth & Bell, 1982). This finding indicates that the product of limited subtilisin digestion of citrate synthase can bind NADH as well as native enzyme can, on a moles per unit weight basis. We have confirmed this conclusion by monitoring NADH binding, by fluorescence enhancement, as a function of time during subtilisin digestion (data not shown). In addition, a sample of the material from the front of the large peak in Figure 4 was titrated with NADH at pH 7.8 in the spectrofluorometer; it gave a K_D value of 1.7 μM and a total binding capacity of 0.74 mol of NADH/47 000 g of protein, essentially the same as those from untreated enzyme (Duckworth & Bell, 1982).

Discussion

The results presented in this paper show that subtilisin interacts with *E. coli* citrate synthase, a large, allosteric enzyme, in much the same way that it does with the small, nonallosteric pig heart enzyme. A principal site of cleavage, located between residues Ala-321 and Val-322 in the pig heart sequence (Bloxham et al., 1980, 1981), is mirrored by a homologous Arg–Val bond in the *E. coli* enzyme. Additional cleavage sites in the *E. coli* citrate synthase are located three and five residues to the N-terminal side of this Arg–Val bond. We have determined the sequence of 19 residues near these sites: this sequence is partially homologous to residues 319–337 in the published sequence of the pig heart enzyme, with nine identities.

Besides this group of three neighboring sites, the *E. coli* enzyme is attacked by subtilisin at two further adjacent positions on either side of a lysine residue to give a short 7.5K fragment. We have determined the sequence of 18 residues, commencing with this lysine, and find a partial homology between this sequence and residues 369 and 386 of the pig heart sequence, with eight identities. No indication was given by Bloxham et al. (1980, 1981) for a subtilisin cleavage site in this area of the pig heart sequence, but they chose digestion conditions so as to keep the products as simple as possible.

The availability of a detailed three-dimensional structure for pig heart citrate synthase allows a plausible explanation for the effect of limited subtilisin proteolysis on the activity of that enzyme. Subtilisin cleavage between residues 321 and 322 would be expected to disturb the structural relationships among the active site residues, since His-320 and Arg-329 are both likely to be involved in the catalytic steps of the reaction, while the peptide backbone of residues 314–320 is involved in binding the adenine ring of acetyl coenzyme A (Remington et al., 1982). The actual site of subtilisin cleavage of the pig heart enzyme is on the surface of the subunit, in a nonhelical loop. Turning to the *E. coli* enzyme, from Figure 3B it can be seen that several of the residues in this critical active site region, including histidine and arginine at positions homologous to 320 and 329, respectively, in the pig heart sequence, are conserved. The region of pig heart sequence homologous to the subsidiary subtilisin sites in the *E. coli* enzyme (Figure 3C) is also a nonhelical loop, and it is also on the surface of the pig heart subunit, very close to the major subtilisin site, in the structure of Remington et al. (1982). One of the residues in this region that is the same in the *E. coli* and pig heart sequences, Asp-375 (pig heart numbering), appears to be involved in catalysis (Remington et al., 1982), while another, Asn-373, lines the citrate binding pocket [Figure 15a of Remington et al. (1982)]. If the folding pattern of the *E. coli* enzyme were the same in the areas of the subtilisin targets as it is in apparently homologous parts of the pig heart enzyme, then these targets would all be close together on the subunit surface. More extensive evidence will be needed to confirm this possibility. At present, however, it is tempting to speculate that the subunits of NADH-sensitive (*E. coli*) and NADH-insensitive (pig heart) citrate synthases share important structural features. In this case, the existence of allosteric properties in the *E. coli* enzyme might be attributed to differences in subunit interactions rather than to subunits of fundamentally different structure.

In the absence of denaturing agents, the proteolytic fragments of *E. coli* citrate synthase elute from Sephadex G-200 slightly ahead of the untreated enzyme (Figure 4). Under the conditions in which this column was run (20 mM Tris-HCl containing 0.05 M KCl), native enzyme behaves as a dimer–hexamer mixture, with a weight-average molecular weight of about 240 000 (Tong & Duckworth, 1975). Since the major components of the leading edge of the main peak in Figure 4 are the cleavage products, it seems likely that the fragments aggregate in a nonspecific manner.

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Registry No. NADH, 58-68-4; citrate synthase, 9027-96-7.

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Topological Localization of Proteolytic Sites of Sodium and Potassium Ion Stimulated Adenosinetriphosphatase†

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ABSTRACT: The (Na^+ and K^+)-stimulated adenosinetriphosphatase [(Na^+,K^+)-ATPase] consists of two different polypeptides, α and β, both of which are embedded in the plasma membrane. The α chain from dog kidney (Na^+,K^+)-ATPase can be hydrolyzed at specific sites by trypsin and chymotrypsin [Castro, J., & Farley, R. A. (1979) *J. Biol. Chem. 254*, 2221-2228]. In order to position these sites with respect to the lipid bilayer, we have treated sealed, inside out vesicles from human red cells and unsealed kidney enzyme membranes with trypsin and chymotrypsin and have used ouabain-stimulated phosphorylation to identify the (Na^+,K^+)-ATPase and its fragments. All of the proteolytic sites observed in the kidney membranes are accessible in the inside out vesicles. The ouabain-inhibitable uptake of $^{86}Rb^+$ in human red blood cells is resistant to externally added chymotrypsin. These results indicate that the proteolytic sites of the (Na^+,K^+)-ATPase are exposed on the cytoplasmic side of the membrane.

The (Na^+,K^+)-ATPase[1] is an intrinsic membrane protein that couples the hydrolysis of ATP to active transport of Na^+ and K^+ across plasma membranes (Cantley, 1981). In all active preparations of the enzyme, two polypeptides are found in equimolar quantities, a catalytic subunit, α, of approximate M_r 100 000, and a glycoprotein, β, of approximate M_r 60 000. The α chain spans the lipid bilayer; it contains the active site for ATP hydrolysis on the cytoplasmic side, and it can be labeled with photoaffinity derivatives of ouabain (Forbush et al., 1978), a specific inhibitor which binds to the extracellular surface of red blood cells (Perrone & Blostein, 1973). The existence of a carbohydrate moiety on the β chain implies that part of the polypeptide lies on the extracellular side of the membrane. The catalytic subunit can be phosphorylated either

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[1] Abbreviations: (Na^+,K^+)-ATPase, sodium and potassium ion stimulated adenosinetriphosphatase; P_i, inorganic orthophosphate; NaDodSO$_4$, sodium dodecyl sulfate; $K_{0.5}$, concentration of ligand that yields a half-maximal amount of product; PAS, periodic acid-Schiff.

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