tified proton donor (see also Rose, 1960) during the phosphoryl transfer from the P-enolpyruvate to ADP. Since the four- and five-carbon homologs act as effective competitive inhibitors and the homologs with greater than five carbons have no apparent inhibitory characteristics, it would appear that the enzyme is capable of distinguishing quite specifically between them, thus, the active site must be quite restrictive. Also, the increased effectiveness of the four-carbon homolog compared with the five-carbon homolog also would appear to support the extreme steric selectivity of pyruvate kinase. In light of these observations, one might postulate that the methyl or the ethyl group attached to the vinyl carbon prevents the attachment of these homologs to the unidentified proton donor site, but does not prevent the attachment of the carboxylate group to the other substrate site (or possibly *vice versa*). The homologs having greater than five carbons and the dimethyl homolog apparently do not bind to the enzyme since no observed effect on the activity of the enzyme was noted. One must, therefore, conclude that the most plausible explanation for the different behavior of the homologs in enzymatic reactions must be stereochemical.

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Subunits, Composition, and Related Properties of Succinyl Coenzyme A Synthetase*

Claus Leitzmann,† Jang-Yen Wu, and P. D. Boyer

ABSTRACT: Succinyl coenzyme A synthetase from *Escherichia coli* (mol wt *ca.* 146,000), both phosphorylated or nonphosphorylated, was dissociated into subunits by exposure to dilute *p*-mercuribenzoate or sodium dodecyl sulfate solutions, to concentrated urea or guanidine hydrochloride solutions, or by alkylation with iodoacetamide or succinylation with succinic anhydride in urea solutions. Considerable aggregation tendency was noted. Subunits were characterized by sucrose density gradient, gel filtration, column chromatography, gel electrophoresis, and ultracentrifugal analyses. Alkylation in urea gave subunits of about 0.5 and succinylated subunits of about 0.25 of the original size. Sedimentation equilibrium measurements indicated possible presence of smaller fragments after both alkylation and succinylation. Complete amino acid analyses are given; tryptophan at approximately eight residues per mole is present in the smallest amount. The results are consistent with presence of at least four subunits of approximately equal size and with similar or possibly identical composition of the phosphorylated and nonphosphorylated subunits. Tests with possible modifiers of catalytic activity showed no prominent effects other than those indicative of competition with substrates. Some improvements in enzyme preparation and a more sensitive catalytic assay are reported. A lack of correlation between capacity for phosphoenzyme formation and catalytic activity of different preparations is confirmed.

Succinyl-CoA synthetase from *Escherichia coli* has been reported to have a molecular weight of approximately 160,000 (Gibson *et al.*, 1967) and of 141,000 (Ramaley *et al.*, 1967). Presence of subunits thus seems probable. Evidence for dissociation of the enzyme by *p*-mercuribenzoate has been mentioned (Ramaley *et al.*, 1967), and merthiolate treatment has been shown to give subunits as measured by immunodiffusion (Grinnell *et al.*, 1969). The enzyme can be phosphorylated by more than one and approaching two phosphoryl groups per mole (Moyer *et al.*, 1967; Grinnell and Nishimura, 1969), suggesting that at least two and possibly more subunits may be present.

The present paper presents some modifications in enzyme preparation, the amino acid composition of the enzyme, and evidence for dissociation of the enzyme into at least four subunits. A considerable tendency for aggregation

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† Postdoctoral Fellow of the Institute of General Medical Sciences, U. S. Public Health Service.

under various dissociation conditions has been noted, and an indication of dissociation into more than four subunits observed. Under some circumstances, separation of phosphorylated from nonphosphorylated subunits is possible.

Experimental Procedures

Purification of Succinyl-CoA Synthetase. The purification procedure as described by Ramaley *et al.* (1967) was used with the following modifications and additions. All of the centrifugations were performed in a refrigerated Sorvall angle centrifuge for 30 min at 17,000 rpm with a SS-34 rotor or at 8500 rpm with a GSA rotor.

Sonic Extraction. Sonications were made in smaller batches. A 200-g portion of the thawed cell paste was suspended in 400 ml of freshly prepared 0.02 M potassium phosphate–0.02 M $MgCl_2$ at pH 7.5 (sonication buffer). Aliquots (150 ml) in a 200-ml beaker (Berzelius tall form) were treated for 10 min with a Bronson sonicator using tap 8. The suspension was stirred with a magnetic stirrer and maintained at 0–5° by immersion in an ethanol–ice bath. Cellular debris and unbroken cells were removed by centrifugation. The supernatant fraction was decanted and the residue fraction was resuspended in 250 ml of the sonication buffer. The suspension was sonically treated for 10 min as described above and centrifuged. The supernatant fraction was combined with the initial decanted fraction and made up to 700 ml with the sonication buffer.

Protamine Sulfate Treatment. To the supernatant solution of the crude extract, 2.5 g of solid protamine sulfate was added with continuous stirring for 15 min. The suspension was centrifuged and the pellet discarded.

The acetone and ammonium sulfate fractionation steps were not altered.

Heat Treatment. Succinate and AMP were present during the heat treatment. The ammonium sulfate precipitate was dissolved in 50 ml of 0.1 M Tris, 0.1 M KCl, 0.1 M succinate, 0.1 M potassium phosphate, and 10^{-5} M AMP at pH 7.2 and heated to 55° in a 70° water bath, then transferred to a 55° bath for 5 min. The heated enzyme was cooled rapidly in an ice–water bath and centrifuged. The precipitate was discarded. The supernatant fluid was put on a 4.5 × 90 cm column, containing Sephadex G-50, medium, and equilibrated with 0.05 M potassium phosphate, 0.05 M KCl, and 10^{-4} M EDTA at pH 7.2 (phosphate–KCl buffer). The enzyme solution was eluted with the same buffer and the protein fractions were pooled.

Chromatography on DEAE-Sephadex. The columns used were larger than previously. The enzyme solution was applied to an 8.0 × 70 cm column of DEAE-Sephadex, equilibrated with the phosphate–KCl buffer. The column was eluted with a linear gradient of 8 l. of phosphate–KCl buffer and 8 l. of the same buffer containing 0.16 M KCl. The fractions containing the enzyme were applied to a 4.5 × 160 cm DEAE-Sephadex column eluted with a total of 8 l. of a linear gradient of the above buffers. The pooled fractions of the enzyme were precipitated with solid ammonium sulfate.

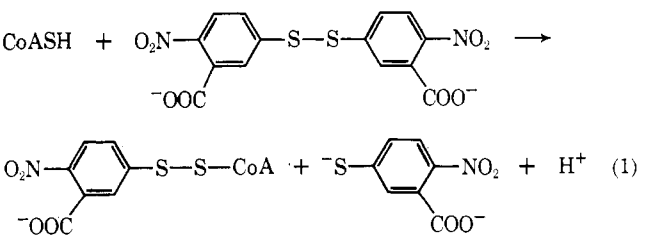
To obtain satisfactory flow rates, the DEAE-Sephadex was washed by suspension and settling successively with 0.1 M NH_4OH, 0.1 M HCl, and 5% ethanol, with five to six suspensions in distilled water after each solution, and with decantation of any fines after each washing.

Chromatography on Hydroxylapatite. Use of a Bio-Gel preparation (Bio-Rad Laboratories) improved flow rates. The enzyme was collected by centrifugation, resuspended in a minimal volume of 0.1 M Tris-Cl (pH 7.2)–0.1 M KCl (Tris-KCl buffer), and passed through a Sephadex G-50 column (4 × 30 cm; coarse beaded form), previously equilibrated with the same buffer. The enzyme solution was put on a 4.5 × 30 cm Bio-Gel HT column that was equilibrated with a Tris-KCl buffer. The enzyme was eluted with a linear gradient of 1 l. of the Tris-KCl buffer and 1 l. of the same buffer containing 10 g of $(NH_4)_2SO_4$ per 100 ml of final volume. Enzyme fractions were pooled and the protein precipitated by addition of solid ammonium sulfate. The precipitate was collected by centrifugation and dissolved in a minimum amount of Tris-KCl buffer.

Sephadex G-200 Gel Filtration. The enzyme solution was introduced into a 4.5 × 175 cm Sephadex G-200 column, equilibrated, and eluted with the Tris-KCl buffer by reverse flow. The fractions containing the enzyme were pooled and kept at −20°. The specific activity of the enzyme preparations varied between 20 and 26 units per mg and represented 40–45% of the activity in the crude extract. *E. coli* cells (200 g) yielded 100–150 mg of succinyl-CoA synthetase.

Preparation of Phosphorylated Enzyme. Enzyme labeled with ^{32}P/[^{32}P]E was prepared by use of conditions described by Ramaley *et al.* (1967). For most experiments, the [^{32}P]E was separated from [^{32}P]P_i by gel filtration on Sephadex G-100.

Enzyme Concentration and Activity Assays. Activity was measured by appearance of absorbancy at 230 mμ due to succinyl-CoA formation (Ramaley *et al.*, 1967), or by assay in the reverse direction with measurement of CoA formation based on the reaction of CoASH with Ellman's reagent (1959), 5,5-dithiobis(2-nitrobenzoic acid) (eq 1). Advantages of



(1)

this assay are that measurements are made at 412 mμ where protein and nucleotide do not absorb, removal of CoA helps maintain linearity with time, and the assay is more sensitive because the mercaptide ion has a molar absorbancy at 412 mμ of 13,600 compared with 4500 for the thio ester bond at 230 mμ. Assay mixtures when testing for possible inhibitors or effectors contained 50 μM succinyl-CoA, 1 mM P_i, 0.1 mM ADP, 10 mM $MgCl_2$, 50 mM Tris-Cl (pH 7.2), 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid), and 1 to 5 μg of the enzyme in a final volume of 1 ml. For rates approaching maximal velocity, substrate concentrations five times the above are appropriate. A concentration of 0.5 mM 5,5′-dithiobis-(2-nitrobenzoic acid) sufficed for a near-maximal rate. With 1 and 2 mM, 15 and 45% inhibition was noted, respectively, and 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) gave 74% of the maximal velocity. Enzyme concentrations were estimated from the A_{280}, and units of enzyme activity were expressed as previously reported (Ramaley *et al.*, 1967).

Amino Acid Analysis. Samples of succinyl-CoA synthetase were dialyzed against distilled water for 24 hr at 4° and then lyophilized. The protein was hydrolyzed under reduced pressure in 5.7 N constant-boiling HCl at 110° for 24, 48, or 72 hr (Moore and Stein, 1963). Analyses were performed with a Beckman-Spinco Model 120B amino acid analyzer, essentially as described by Spackman *et al.* (1958).

The half-cystine and methionine contents were determined from the amount of cysteic acid and methionine sulfone formed, respectively, after performic acid oxidation (Moore, 1963) and acid hydrolysis of triplicate samples. Tryptophan was estimated spectroscopically as described by Edelhoch (1967).

Isoelectrofocusing. The isoelectric point of the enzyme was determined at 4° by the isoelectric focusing technique of Vesterberg and Svensson (1966) using a column and ampholytes from LKB. The enzyme (10 mg) was exposed to 300 V for 64 hr.

Polyacrylamide Gel Electrophoresis. The method of Ornstein (1964) was followed, using a 3.5% gel and carrying out all operations in a 4° cold room. The gels were cooled by an air stream to minimize local heating. Electrophoresis was carried out at pH 9.5 in Tris-glycine buffer (3.0 g of Tris, 14.4 g of glycine per l.) with a currect of 5 mA/gel for 90 min. The gels were treated with Amido-Schwarz and unbound dye was removed by electrophoresis in 7% acetic acid at 8 mA/gel. ^{32}P bound to protein was determined by slicing the gel into short sections, dispersing in 95% ethanol, drying, and counting.

Labeling of Succinyl-CoA Synthetase with [^{14}C]Succinic Acid. The enzyme was labeled with [^{14}C]succinate by taking advantage of the observation of Robinson *et al.* (1969) of an irreversible succinylation that occurs with catalytically active enzyme in the presence of labeled succinate and other substrates. The excess ^{14}C substrates were removed by Sephadex G-50 gel filtration. This simple procedure gives a convenient method of preparing catalytically active enzyme labeled with ^{14}C.

Treatment with p-Mercuribenzoate or Dodecyl Sulfate. Solutions of the Na salt of *p*-hydroxymercuribenzoate, adjusted to pH 8 with HCl, were added with stirring to 35 to 140 μM solutions of enzyme in 0.1 M Tris-Cl buffer pH 7.2, to give 0.7 to 14 mM final *p*-mercuribenzoate. Alternatively, *p*-mercuribenzoate was added by dialysis. For treatment with dodecyl sulfate, the solid sodium salt was added in small increments to approximately 70 μM enzyme in 0.1 M Tris-Cl buffer, pH 7.2, to give a final concentration of 35 mM sodium dodecyl sulfate, with or without 100 mM 2-mercaptoethanol present.

Treatment with Iodoacetamide or Succinic Anhydride. Enzyme preparations for alkylation or succinylation were first exposed to 8 M urea and 0.1 M mercaptoethanol as indicated elsewhere. Alkylation with iodoacetamide was performed essentially as described by Crestfield *et al.* (1963). Solid iodoacetamide was gradually added at room temperature to the stirred urea solution to give a final iodoacetamide concentration of 0.09 M. The pH was maintained at 8.2 by addition of 1 N NaOH. The procedure required about 1 hr. The presence of mercaptoethanol in slight excess of the alkylating agent is in accord with the procedure of Crestfield *et al.* (1963) with iodoacetate and of Weber (1968) with iodoacetamide.

Treatment with succinic anhydride was performed essentially as described by Habeeb *et al.* (1958). Solid succinic anhydride was added slowly to a stirred solution of the enzyme over a period of about 40 min at room temperature. The pH was kept close to 8.2 by the addition of 1 N NaOH. About 16 molecules of anhydride was added for every lysine residue present in the protein. After standing for 3 hr at room temperature, the solution was dialyzed as indicated elsewhere.

Treatment with Gd·HCl.[1] Solid Gd·HCl, Mann Ultra Pure grade, was added to a carefully stirred solution of enzyme in 0.1 M phosphate buffer (pH 7.4) containing 0.1 M 2-mercaptoethanol and 0.1 mM EDTA. The final Gd·HCl concentration was 6 M. After 3 hr at room temperature, the solution was dialyzed overnight at 4° against 6 M Gd·HCl containing 0.1 M 2-mercaptoethanol and 0.1 mM EDTA. Sedimentation velocity was measured with protein concentrations of 3.0, 6.0, 7.5, 9.0, and 12.0 mg per ml.

Analytical Ultracentrifugation. Sedimentation velocity experiments were measured with a Spinco Model E analytical ultracentrifuge using a 30-mm double-sector cell, schlieren optics, and speeds of 50,740 or 48,000 rpm at 20° unless otherwise mentioned. Sedimentation equilibrium runs were made with the use of an electronic speed control and Raleigh interference optics. The high-speed sedimentation equilibrium measurements were made with a six-channel cell as described by Yphantis (1964) at 7–14°. Runs were continued until no further change in the distribution of concentration in the cell occurred (at least 40 hr). Sapphire windows were used to minimize the optical distortion. Plates were read in a Nikon microcomparator with a 50-fold magnification lens. Sedimentation coefficients were corrected to standard conditions measurements at several protein concentrations and with use of usual formulations (Schachman, 1959, eq 26, p 82). For measurements with succinylated or alkylated enzyme, or enzyme in Gd·HCl solution, the partial specific volume of the native enzyme was used as a reasonable approximation. Viscosities were measured with a Ubbelhode viscometer and densities with a pycnometer at 20 ± 0.1°.

Sucrose Gradient Centrifugation. The experimental procedure described by Martin and Ames (1961) was followed for the handling of sucrose gradients, using a Büchler density gradient sedimentation system with a triple outlet gradient mixer. All gradients were of 5 to 20% sucrose in 0.1 M Tris-Cl–0.1 M KCl buffer at pH 7.2. Enzyme preparations were applied to the gradient in 0.1-ml volumes. After centrifugation at 40,000 rpm for 16 hr at 4° with the SW50 L rotor in a Model L Spinco centrifuge, 35 drop fractions were collected by a Büchler fraction collection device. Each gradient yielded an average of 380 drops.

Results

Enzyme Purification. Both the specific activity (15–32 units/mg) and yield of total activity (8 to 50 units per g of fresh cells) have been noted to change from one batch of *E. coli* to the next, and the purification procedure may need to be altered somewhat for each new harvest of *E. coli*.

A shorter sonication time with smaller amounts of orga-

[1] Gd·HCl = guanidine hydrochloride.

TABLE I: Amino Acid Composition of Succinyl-CoA Synthetase.

Amino Acid	Residues/mole[a]	Weight % of Amino Acid	Residues per 100 Total Residues
Lysine	90.5 ± 1.1	8.04	6.57
Histidine	22.2 ± 0.2	2.08	1.59
Arginine	41.1 ± 0.1	4.41	2.96
Aspartic acid[b]	111.2 ± 4.8	8.81	8.01
Threonine[c]	84.0 ± 0.2	5.89	6.06
Serine[c]	43.0 ± 0.1	2.58	3.10
Glutamic acid[b]	147.7 ± 0.3	13.19	10.68
Proline	58.4 ± 0.3	3.88	4.18
Glycine	172.6 ± 1.6	6.80	12.48
Alanine	159.2 ± 0.5	7.79	11.47
Half-cystine[d]	26.7	1.92	1.95
Valine[e]	126.2 ± 0.7	8.61	9.09
Methionine[d]	27.6 ± 0.5	2.53	2.02
Isoleucine[e]	99.9 ± 0.9	7.79	7.22
Leucine[e]	102.2 ± 0.3	7.95	7.36
Tyrosine	25.8 ± 0.5	2.92	1.88
Phenylalanine	39.0 ± 0.4	3.96	2.81
Tryptophan[f]	8.2 ± 0.2	1.03	0.58

[a] Based on molecular weight of 146,000. [b] Figures included both free and amidated residues. [c] Extrapolated to zero time of hydrolysis. [d] From hydrolysate of performic acid oxidized preparation. [e] Values are taken from the hydrolysate of 72-hr hydrolysis. [f] Estimated from ultraviolet absorption by the method of Edelhoch (1967).

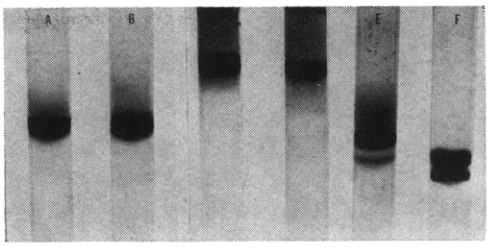


FIGURE 1: Polyacrylamide gel electrophoresis. Sampes were separated on polyacrylamide gel as described under Experimental Procedures. For samples C–F, 15 to 30 mg of enzyme was exposed to 8 M urea for 4 hr at room temperature in 0.1 M Tris-HCl buffer, pH 7.2. Preparations were then treated with iodoacetamide or succinic anhydride as described under Experimental Procedures: (A) enzyme (100 μg) as isolated after gel filtration step; (B) enzyme as A but phosphorylated by [^{32}P]/P_i in presence of succinyl-CoA; (C) enzyme (200 μg) dissociated by urea–iodoacetamide treatment; (D) [^{32}P]enzyme (100 μg) dissociated by urea–iodoacetamide treatment; (E) enzyme (100 μg) dissociated by urea–succinic anhydride treatment; (F) [^{32}P]enzyme (150 μg) dissociated by urea–succinic anhydride treatment.

nisms improved the initial yield considerably. This treatment resulted in a higher protein concentration in the supernatant solution, but this protein could be eliminated in the following fractionation steps without any apparent disadvantages.

The protamine sulfate treatment removed some proteins along with most of the nucleic acids. Although protamine sulfate interferes with the determination of the enzyme activity at 230 mμ, subsequent precipitation separates the protein from protamine sulfate.

The heat treatment performed in the presence of ammonium sulfate, succinate, and AMP seemed to protect the enzyme better against denaturation. Little enzyme activity was lost but 40% of the protein was removed.

A distinct improvement of the purification procedure was obtained by use of Sephadex G-200 gel filtration step. A rather long column was needed and reverse flow helped maintain a reasonable flow rate. The enzyme before gel filtration showed two distinct minor contaminants upon gel electrophoresis that were removed by gel filtration. Gel electrophoresis patterns for the purified enzyme as isolated and the corresponding phosphorylated enzyme are shown in Figure 1A and -B, respectively. The enzyme also appeared homogeneous in ultracentrifugation.

Amino Acid Analysis and Isoelectric Point. The amino acid composition of the purified succinyl-CoA synthetase is shown in Table I. The first column gives the moles of amino acid per mole of enzyme calculated on the assumption that the enzyme contained only amino acids and that all amino acids were recovered by the techniques used. Also listed in the Table are the number of residues of each amino acid per 100 residues present and the weight per cent of each amino acid.

Values for the weight per cent of the amino acids (Table I) were used to calculate the partial specific volume of the enzyme (Cohn and Edsall, 1943). The value obtained, 0.74, was slightly higher than the value of 0.73 used previously in molecular weight estimations (Ramaley *et al.*, 1967), leading to a revised molecular weight of 146,000.

The pI estimated by isoelectric focusing was 5.5. The enzyme is quite unstable at pH values below 6.0 (Ramaley *et al.*, 1967) and only a small part of the initial activity could be recovered from the isoelectric focusing experiment. The observed value of 5.5 determined by isoelectric focusing is in harmony with the high content of the acidic amino acids.

Dissociation by p-Mercuribenzoate and Sodium Dodecyl Sulfate. In confirmation of the observation of Ramaley *et al.* (1967), succinyl-CoA synthetase was dissociated by *p*-mercuribenzoate. However, treatment of the enzyme with *p*-mercuribenzoate whether by direct addition or by dialysis (see Experimental Procedures) gave heterogeneous mixtures, with considerable aggregated high molecular weight material apparent upon ultracentrifugal examination. A portion of the enzyme (up to 50%) did show a decreased sedimentation coefficient (s = 2.3 S compared with 6.2 S for the native enzyme), indicative of possible dissociation. Dissociation was also apparent from behavior in sucrose density gradients, as shown in Figure 2. This figure gives density gradient patterns for native enzyme that is irreversibly labeled with [^{14}C]-succinate (Robinson *et al.*, 1969) but still shows catalytic activity and for [^{14}C]succinyl- or ^{32}P-labeled enzyme treated with *p*-mercuribenzoate. Separation of both the ^{14}C- and ^{32}P-labeled material after treatment with the mercurial shows that most of the enzyme formed similar, lower molecular weight components.

Exposure of the enzyme to 35 mM sodium dodecyl sulfate

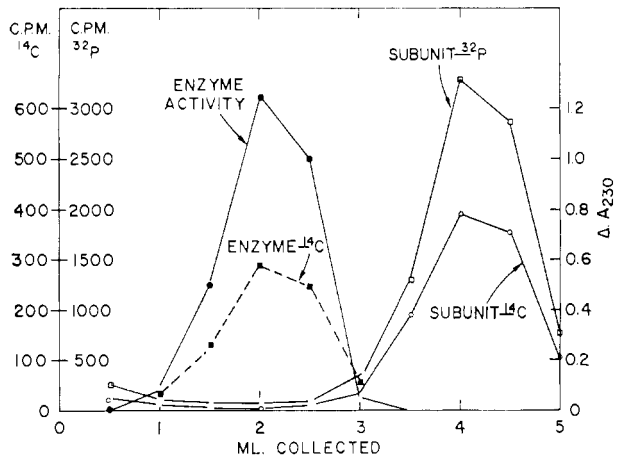


FIGURE 2: Separation of enzyme dissociated with *p*-mercuribenzoate from native enzyme on a sucrose density gradient. Separate samples of [^{14}C]succinate or ^{32}P-labeled enzyme at concentrations of 5 mg/ml were treated with 1.8 mM *p*-mercuribenzoate in 0.1 M Tris-HCl buffer at pH 7.2 for 5 hr at room temperature. Aliquots of 0.1 ml of the native or *p*-mercuribenzoate-treated protein containing about 0.5 mg of protein were layered on top of the gradients and separated as described under Experimental Procedures.

gave products which separated on sucrose density gradients like the *p*-mercuribenzoate-treated preparations.

When phosphorylated enzyme was treated with *p*-mercuribenzoate, and subsequently separated on DEAE-Sephadex, both total protein (as measured by A_{280}) and protein-bound ^{32}P migrated close together and were eluted considerably before intact [^{32}P]enzyme as noted in Figure 3. However, the specific activity of the protein in the subunit peak varied considerably throughout the peak, and indicated greater heterogeneity of the nonphosphorylated than of the phosphorylated components.

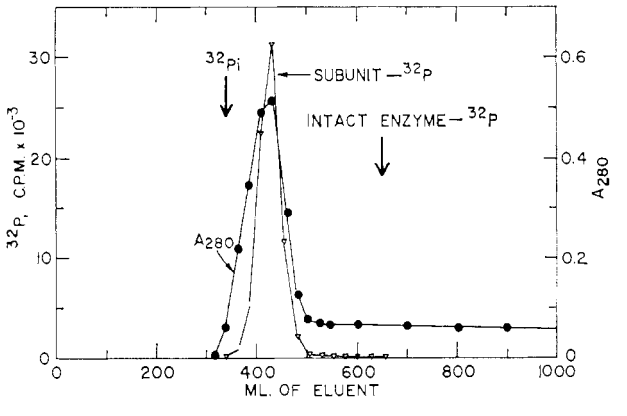


FIGURE 3: Separation of enzyme dissociated with *p*-mercuribenzoate on a DEAE-Sephadex column. A solution containing 4 mg of [^{32}P]enzyme per ml was dialyzed against a buffer containing 3.4 mM *p*-mercuribenzoate in 0.05 M potassium phosphate, 0.05 M KCl, and 10^{-4} M EDTA at pH 8.0 for 24 hr at 4°. The sample was applied to a 2.2 × 50 cm column of DEAE-Sephadex and eluted with a linear gradient of 500 ml of the above buffer at pH 7.2 and 500 ml of the same buffer with 0.16 M KCl. The positions of migration of intact [^{32}P]enzyme and of [^{32}P]P_i on the column are indicated by the arrows.

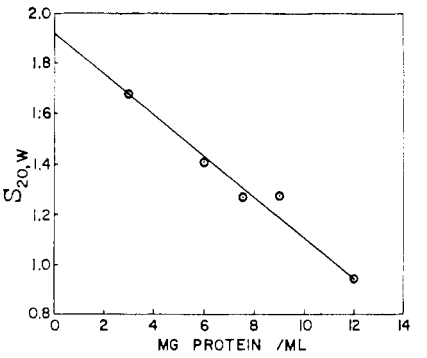


FIGURE 4: Sedimentation velocity of succinyl-CoA synthetase in 6 M Gd·HCl, pH 6.4, containing 0.1 M 2-mercaptoethanol and 0.1 mM EDTA. (See Experimental Procedures for details.)

Dissociation by Urea of Gd·HCl. Enzyme exposed to 8 M urea or 6 M Gd·HCl in the presence of 0.1 M mercaptoethanol and 0.1 M phosphate buffer (see Experimental Procedures) and examined in the ultracentrifuge in these media showed a markedly decreased sedimentation coefficient. Similar dissociation patterns were observed whether the enzyme was exposed only a short period or up to 5 hr at room temperature. In all instances, unexpected formation of aggregated products, estimated as 20 to 30% of the total enzyme, was observed. The soluble products did, however, show a single, apparently homogeneous, peak. Figure 4 gives data for the protein in Gd·HCl solutions. From extrapolation of plots of $s_{20,w}$ against concentration to $c = 0$, the $s^0_{20,w}$ value is approximately 1.9 S.

The partial aggregation of the enzyme observed in 6 M Gd·HCl in presence of 0.1 M mercaptoethanol and 0.1 mM EDTA at pH 6.4 appears somewhat unusual. As noted by Tanford (1968), polypeptide chains in high concentrations of guanidine hydrochloride behave as random coils. Mention

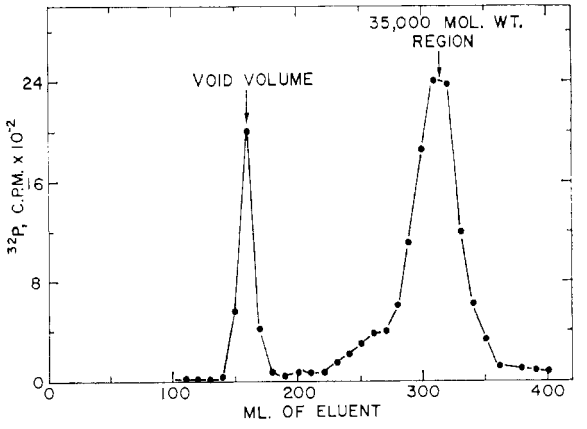


FIGURE 5: Separation of enzyme dissociated by exposure to urea and succinic anhydride on Sepadex G-200. ^{32}P-Labeled enzyme in 0.1 M Tris-KCl buffer, pH 7.2, was exposed to 8 M urea and 0.1 M mercaptoethanol for 5 hr at room temperature then succinylated as described under Experimental Procedures. A 1-ml aliquot of the preparation, containing about 5 mg of enzyme, was separated on a 2 × 120 cm column of Sephadex G-200. With column equilibration and elution by 0.1 M Tris-Cl-0.1 M KCl, pH 7.2, at 4°, the flow rate was about 10 ml/hr.

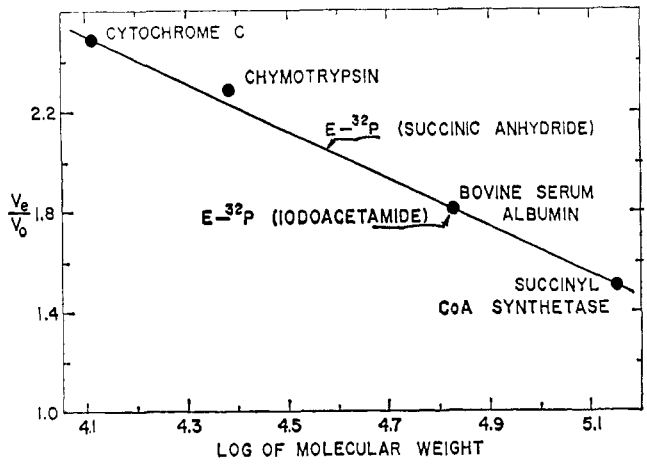


FIGURE 6: Molecular weight estimations by Sephadex G-200 filtration. Solutions of about 1 ml, containing 1 mg of proteins of known molecular weight or samples prepared as described in the text, were separated as described with Figure 5. The circles indicate the position of peak midpoints for samples of known molecular weights. The arrows indicate the position of migration of the enzyme dissociated by urea–iodoacetamide or urea–succinic anhydride treatment.

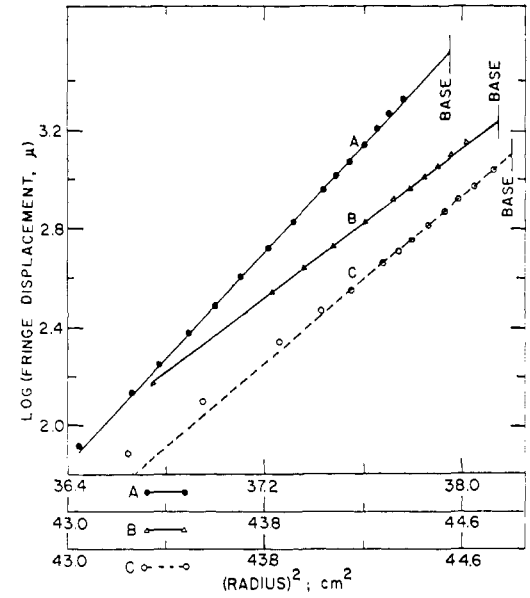


FIGURE 7: Ultracentrifugal sedimentation equilibrium characterizations of dissociated enzyme preparations. The plots show the log of the fringe displacement in microns againt r^2. (A) Succinyl-CoA synthetase, 10 mg/ml in 0.1 M Tris-KCl buffer at pH 7.2, was exposed to 8 M urea and 0.1 M mercaptoethanol for 4 hr at room temperature, succinylated with succinic anhydride as described under Experimental Procedures, and then dialyzed overnight at 4° against 0.1 M Tris brought to pH 12.5 by the addition of NaOH. Centrifuged 45 hr, protein concentration 0.8 mg/ml, 34,000 rpm, 7.1°. (B) Conditions were similar to A, but ^{32}P-labeled enzyme was used and the final dialysis was against 0.1 M Tris-Cl buffer, pH 7.2. Aggregated material was removed by passage through Sephadex G-100, with use of the ^{32}P label to detect protein fractions. Centrifuged 44 hr, protein concentration 0.3 mg/ml, 26,000 rpm, 10.0°. (C) Conditions similar to A, but the preparation was alkylated as described under Experimental Procedures prior to succinylation and the final dialysis buffer at pH 7.2 contained 10^{-4} M EDTA. Aggregated products were removed as in B. Centrifuged 48 hr, protein concentration 0.2 mg/ml, 34,000 rpm, 14.0°.

may be made that de Crombrugghe *et al.* (1966) have reported that thyroglobulin reduction by 2-mercaptoethanol in 6 M Gd·HCl is considerably hindered at pH 7.95 as compared with treatment in 8 M urea at pH 10.1. Also, Butzow (1968) has reported that fungal *p*-diphenol oxidase is only partially dissociated in 6 M Gd·HCl containing 0.1 M mercaptoethanol.

Dissociation by Treatment with Urea and Succinic Anhydride. The enzyme when exposed to urea then treated with an excess of succinic anhydride gave some aggregated material accompanied by conversion of most of the enzyme into subunits. The separation pattern on a Sephadex G-200 column observed with phosphorylated enzyme following such treatment is shown in Figure 5. Some of the radioactivity appeared with the void volume, as anticipated for high molecular weight aggregated material. The presence of ^{32}P showed that the aggregated material was derived from the total enzyme protein or the phosphorylated subunit. In similar experiments in which phosphorylated enzyme was succinylated with [^{14}C]succinic anhydride, approximately equal ratios of ^{32}P:^{14}C were observed in both the high and low molecular weight region. In addition, as described later, a similar succinylation followed by dialysis at pH 12.5 avoided aggregation and gave material that migrated as a single peak on Sephadex G-200. Thus, the aggregated material observed in Figure 5 appears to be representative of intact enzyme. As most of the radioactivity appears with the low molecular weight fraction (Figure 5) and as a single peak, both phosphorylated and nonphosphorylated subunits appear to be of approximately the same size. The shoulder on the peak in Figure 5 could represent some undissociated or partially dissociated enzyme.

Molecular weight estimations for the succinylated or alkylated subunits from gel filtration studies seemed plausible because subunits obtained by the procedures used might be expected to resemble globular proteins on filtration behavior. This expectation was verified by estimations of the frictional coefficient of succinylated preparations from sedimentation equilibrium and sedimentation velocity and the relation (see Schachman, 1959), $f = M(1 - \bar{v}\rho)/NS$. From the frictional coefficient (6.3×10^{-8} g/sec), the estimated axial ratio (see Tanford, 1963) was 8.5. This value is well within the range for "globular" proteins.

The designation of the peak for the subunits in Figure 5 as the 35,000 molecular weight range, is based on calibrations of the column with proteins of known molecular weight. Data from such calibrations are shown in Figure 6, where the points represent the midpoint of the peaks for the various proteins. In accord with Whitaker (1963) and Andrews (1964), the procedure gives a relatively good straight-line relationship thus giving some confidence in the molecular weight estimations.

The molecular weight of the ^{32}P-labeled fragments produced by the urea succinylation treatment, as described with Figure 5, was estimated by ultracentrifugal measurements. Aggregated products were separated from the low molecular weight fraction by Sephadex G-100 filtration. Sedimentation equilibrium measurements as shown in Figure 7B indicated a homogeneous preparation of molecular weight about 40,000. If all the lysine residues of the protein are succinylated,

TABLE II: Enzyme Phosphorylation Compared with Specific Activity.[a]

Specific Activity of Enzyme Preparation	Phosphorylation of Enzyme	
	By P_i	By ATP
	Phosphoryl Groups/mole	
4.0	1.00	1.34
5.1	0.54	1.17
12.1	1.15	1.38

[a] Samples (1–3 mg) of enzyme were exposed to [^{32}P]P_i and succinyl-CoA or to [^{32}P]ATP under conditions as described by Ramaley *et al.* (1967).

the succinyl groups would be sufficient to contribute about 2000*g* to each of four similar subunits. This leads to a value of 38,000 corrected for succinylation. The results suggest ^{32}P-labeled subunits representing a quarter of the molecule. The slightly higher apparent molecular weight than obtained with gel filtration techniques could reflect traces of aggregated material.

The aggregation noted for the experiment reported in Figure 5 was avoided when a preparation was similarly treated, but dialyzed overnight at pH 12.5 at 4°. Material prepared in this manner was used for ultracentrifugal analysis, as shown in Figure 7. The dissociated protein appeared to be homogeneous, and the molecular weight calculated from the data of Figure 7, as outlined by Yphantis (1964), was 37,000. Thus, the molecular weight of the subunit corrected for contribution of succinyl groups is about 35,000; a figure in good agreement with the estimation from the gel filtration technique. The low temperature of exposure to pH 12.5, the apparent ultracentrifugal homogeneity of the product, and the similar molecular weight to that of the succinylated protein indicated that peptide-bond cleavage did not result from the pH 12.5 exposure.

The urea succinylation treatment was observed to give products from both the enzyme as isolated and the ^{32}P-labeled enzyme that were separable on polyacrylamide gel electrophoresis, as noted in Figure 1E,F. With the enzyme as isolated, a small, more rapidly moving component was observed. In a series of runs with the ^{32}P-labeled enzyme, a similar, more rapidly moving band comprising about 25 to 50% of the total protein was observed (Figure 1F). This band carried nearly all the ^{32}P, demonstrating clear separation of phosphorylated and nonphosphorylated subunits. The small amount of a more rapidly moving band noted with the enzyme as isolated (Figure 1E) could represent that portion of the enzyme that is already phosphorylated in *E. coli*. The tendency for a phosphorylated form to survive the preparation procedure has been reported (Ramaley *et al.*, 1967).

Dissociation by Urea and Alkylation. Treatment of the ^{32}P-labeled enzyme with iodoacetamide in the presence of urea and mercaptoethanol as described under Experimental Procedures gave a distinctly different subunit pattern. A greater tendency for aggregation was observed than with succinylation. As noted in Figure 6, the soluble material obtained migrated on a Sephadex G-200 column in a region corresponding to about 70,000 molecular weight. Thus, the urea alkylation treatment appears to cleave the original enzyme into halves.

The alkylated enzyme or alkylated phosphorylated enzyme migrated as a single band on polyacrylamide gel electrophoresis, as shown in Figure 1C and -D, respectively. Migration of the alkylated enzyme was considerably slower than the succinylated enzyme (Figure 1E,F), as might be anticipated for the extra negative charges introduced by succinylation.

Dissociation by Both Alkylation and Succinylation. Enzyme in urea treated with both succinic anhydride and iodoacetamide, as described in the previous sections, still showed upon ultracentrifugal analysis about 20 to 30% of the protein as aggregated products and an apparently homogeneous soluble material. After separation of aggregated material by Sephadex G-100 filtration, the soluble material was used for equilibrium sedimentation analyses. As noted in Figure 7, line C, the plot of the log of the fringe displacement against r^2 shows a deviation from linearity, indicative of some heterogeneity with higher molecular weight material present. Thus, the molecular weight estimation from the slope of the plot may be too high. The value obtained, after correction for the approximate weight of the succinyl groups, was only about 30,000. This suggests consideration of the possibility that chain rupture or dissociation into units smaller than 0.25 of the original enzyme might have occurred.

Correlation of Phosphorylation and Enzyme Activity. In an earlier report from this laboratory, it was noted that capacity for phosphorylation did not necessarily parallel catalytic activity of different enzyme preparations of relatively high specific activity. In a subsequent study by Grinnell and Nishimura (1969), a correlation of activity with capacity for phosphorylation was observed with enzyme preparations having a specific activity of 6 to 12, expressed in units as used herein. Some additional data are reported here with enzyme preparations of somewhat lower specific activity than previously used by Ramaley *et al.* (1967). Measurements were made of phosphoenzyme formation and activity and of phosphorylation by exposure to either [^{32}P]P_i and succinyl-CoA, or to [^{32}P]ATP. Results presented in Table II show clearly a poor correlation between capacity for phosphorylation from either the P_i or ATP side and the catalytic activity. In addition, they show that with these preparations the phosphorylation from the ATP side was less than that from the P_i side, a result in contrast with the previous observations with preparations of higher activity (Ramaley *et al.*, 1967).

Possible Modifiers of Catalytic Activity. The apparent complexity of the succinyl-CoA synthetase structure suggests that control phenomena resulting from interactions with appropriate metabolites or coenzymes might occur. Tests of a number of substances were made using the assay based on CoA release from succinyl-CoA and with limiting amounts of substrate present to increase the sensitivity of any inhibitors. The results are given in Table III. An inhibitory effect of citrate was readily overcome by addition of an equivalent concentration of Mg^{2+}. The four-carbon dicarboxylic acids of the citric acid cycle showed moderate inhibitions. The inhibition by ATP was overcome by addition of ADP or P_i. Acetyl-CoA showed definitive inhibition, but the concen-

tration used was 34 times that of the succinyl-CoA concentration.

In other related tests using the assay based on succinyl-CoA formation, 0.1 mM δ-aminolevulinic acid, 0.2 mM heme, or up to 2.5 mM cytochrome *c* were without inhibitory effect.

Discussion

The results establish that succinyl-CoA synthetase as isolated from succinate-grown *E. coli* can be dissociated into subunits by exposure to *p*-mercuribenzoate, sodium dodecyl sulfate, or Gd·HCl, or by succinylation or alkylation in urea solution. Exposure to urea and succinic anhydride suffices to give dissociation into at least quarter-molecules, but exposure to urea and iodoacetamide gives a product that behaves as a half-molecule. Tendency for the aggregation complicated the dissociation studies. Two results give confidence that the soluble dissociated products represent components as present in the original enzyme and not as derived from a particular subunit or subunits. These are the appearance of both ^{32}P from phosphorylated enzyme and ^{14}C from succinic anhydride treated enzyme in about equal portions in aggregated and soluble fractions and the obtaining of soluble fragments in amounts considerably greater than half the total original protein.

The clear separation on polyacrylamide gels of the phosphorylated and nonphosphorylated subunits from the succinic anhydride treated preparation suggests the possibility that the enzyme may contain subunits of two distinct types. In all other tests, the phosphorylated and nonphosphorylated subunits appeared identical. The separation by gel electrophoresis could reflect differences resulting from the presence of the phosphorylated histidine residue. This would contribute a slight charge difference, and, in addition, could modify the nature and extent of reaction of groups with succinic anhydride. A small difference in amino acid composition or other modification, as has been observed with aldolase (Lai, 1968), must also be considered.

The possibility exists that the enzyme might be further dissociated into smaller fragments, as indicated by limited ultracentrifugal studies of the enzyme exposed to iodoacetamide and succinic anhydride in urea. The slight curvature of the plot given in Figure 7, line C, is indicative of size heterogeneity of the preparation. The weight-average molecular weight was estimated to be about 32,000 but the number-average molecular weight was only approximately 25,000. Additional studies would be necessary, however, before accepting present evidence as showing the presence of noncovalent or disulfide-bound subunits less than 0.25 the size of the original molecule.

A troublesome and unexplained aspect of studies with the enzyme is the variation encountered in catalytic capacities of various preparations. Not only is there considerable variation in activities of preparations from different laboratories (see Ramaley *et al.*, 1967, for example), but within the same laboratory. Preparations of specific activity as high as 32 were previously obtained in our laboratory (Ramaley *et al.*, 1967), but in the present work preparations which were demonstrably of equal or greater homogeneity had activity of only 20 to 26 units per mg. Enzyme of highest specific activity may not necessarily be closest to the native enzyme in characteristics. For example, modifications leading to loss of control could increase activity. Additional vagaries of the enzyme are apparent from the lack of correlation between the extent of phosphorylation and the catalytic activity noted in this and earlier studies (Ramaley *et al.*, 1967), and the difference in extent of phosphorylation of the enzyme from the P_i and the ATP side. Possible explanations include presence of undetected contaminants or subtle conformational or chemical changes. An enzyme that has lost some catalytic activity might still undergo a slow phosphorylation. An adequate understanding of these differential inactivations of the various reactions of the overall catalysis, although potentially quite interesting, may be difficult to attain. Indeed, the uncontrolled vagaries of the enzyme have discouraged further in depth studies of its structure–function relationships at the present time.

With respect to tests for possible modifiers of the catalytic activity, the inhibitions noted for the four-carbon dicarboxylic acids could result from competition with the binding of succinyl-CoA or possibly with P_i or ADP. Oxalacetate does show, however, a more pronounced effect than succinate.

TABLE III: Effect of Various Compounds on the Activity of Succinyl-CoA Synthetase.[a]

Compound	Concn (mM)	Inhibn (%)	Remarks
Citrate	15	50	Overcome by 15 mM Mg^{2+}
Pyruvate	2	0	
α-Ketoglutarate	10	0	
Succinate	20	45	
Fumarate	20	60	Not overcome by 5 mM ADP
Malate	10	20	
Oxalacetate	10	65	Not overcome by 100 mM P_i, 5 mM ADP, or 50 mM Mg^{2+}
Glutamate	1	0	
Aspartate	10	0	
TPN	9	0	
DPN	10	0	
DPNH	6	0	
AMP	10	15	
ATP	3	50	Overcome by 3 mM ADP or 1 mM P_i, not by 50 mM Mg^{2+}
UTP	10	0	
ITP	10	0	
GMP	10	0	
GDP	6	0	
GTP	4	0	
			Showed about 10% activation
Acetyl-CoA	1.7	50	

[a] Activity was measured by the succinyl-CoA cleavage procedure with limiting substrates present (see Experimental Procedures). The highest concentration tested is given.

Similarly, additional experiments would be necessary to find if the inhibition by ATP and by acetyl-CoA might result from competition with substrates or from interaction at specific sites for the inhibitory substance. It may be pertinent that these experiments are performed with enzyme, from *E. coli* grown in presence of succinate. The enzyme might have different properties when cells are grown on glucose or other carbon sources.

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