Crystallization of Succinyl-CoA Synthetase from *Escherichia coli*

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William T. Wolodko, Michael N. G. James, and William A. Bridger

From the Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7

Well formed, tetragonal prisms of succinyl-CoA synthetase from *Escherichia coli* have been crystallized at room temperature from ammonium sulfate and mixtures of sodium and potassium phosphates. A systematic survey of the conditions for crystallization of the enzyme has been carried out. This has shown the addition of a small amount of an organic solvent (acetone, 2-methyl-2,4-pentanediol, tert-butyl alcohol, or tert-amyl alcohol) to the phosphate media and of CoA to the sulfate media to be beneficial in producing large, single crystals suitable for analysis by X-ray diffraction methods. Preliminary examination of precession photographs reveals that the crystals from phosphate media have a unit cell of symmetry P4,22 with dimensions \(a = b = 94 \, \text{Å}\) and \(c = 248 \, \text{Å}\). Evidence suggests that there may be only half of the \((\alpha \beta)_2\) tetramer/asymmetric unit in these crystals. The crystals from ammonium sulfate media have unit cell dimensions of \(a = b = 99 \, \text{Å}\) and \(c = 399 \, \text{Å}\), a space group of \(P4,22\) (P4,22), and one tetramer/asymmetric unit. They diffract to a resolution of 3.4 Å. Both crystal types have large solvent contents of about 65% of the unit cell volumes. A parameter called "quality index" is introduced to facilitate comparison of crystals grown under a variety of conditions with respect to their quality of X-ray diffraction.

Succinyl-CoA synthetase catalyzes a step of the tricarboxylic acid cycle and thus performs a vital function in aerobic metabolism. The enzyme purified from *Escherichia coli* (EC 6.2.1.5) catalyzes the following reversible reaction.

\[
\text{Mg}^{2+} + \text{Succinyl-CoA} + \text{ADP} + \text{P} \rightarrow \text{Succinate} + \text{CoA} + \text{ATP}
\]

In the forward direction as written, this reaction represents the "substrate level" phosphorylation step of the tricarboxylic acid cycle.

This enzyme is a tetramer with a molecular weight of \(1.4 \times 10^6\) and consists of two types of subunits thought to be assembled as a dimer of \(\alpha \beta\)-dimers (1, 2). Several lines of evidence suggest that the complete active site may be located at the region of contact between the \(\alpha\) and \(\beta\) subunits. Whereas the \(\alpha\) subunit binds ATP and contains the active site histidine residue that is phosphorylated as a catalytic intermediate (3), the \(\beta\) subunit contains sites for the attachment of the substrates succinate and CoA (4, 5). An interesting property of the active site is that it is most competent for catalysis of partial reactions only when all substrate-binding sites are occupied, a catalytic property known as "substrate synergism" (6). The proposed location of the active site at the \(\alpha \beta\) contact provides an obvious rationale for the presence of both subunits in the active enzyme. Less clear, however, is the rationale for the presence of two copies of each subunit in the whole enzyme, especially in light of the tendency of succinyl-CoA synthetase from eukaryotic sources and Gram-positive bacteria to exist as an \(\alpha \beta\)-dimer with a molecular weight of approximately \(7 \times 10^5\) (7, 8). In principle, one would expect to find two active sites/tetramer, but succinyl-CoA synthetase from *E. coli* displays half of the sites reactivity with respect to its phosphorylation by ATP; only one phosphoryl group is observed to be incorporated at any time (9, 10). This manifestation of apparent asymmetry from otherwise symmetric units has prompted several recent studies (2, 11–13), the results of which indicate the operation of catalytic cooperativity between alternating active sites on the \(\alpha \beta\)-dimers; that is, interaction of substrate (particularly ATP) with one active site promotes catalytic events at the other site, mediated through some reciprocal change in conformation of the two halves of the enzyme molecule. One way to reconcile the catalytic properties of this enzyme with its quaternary structure would be to have a clear representation of the conformation of the tetrameric enzyme in three dimensions. This study is the first step toward that end. In this paper, we report the crystallization and the preliminary crystallographic data of single crystals of succinyl-CoA synthetase from *E. coli*.

**EXPERIMENTAL PROCEDURES**

**Materials**—Succinyl-CoA synthetase was purified from *E. coli* (Crooks strain) grown on a phosphate-buffered, succinate-based medium as described previously (14, 15). To ensure maximum phosphorylation of the purified enzyme, the preparation was subjected to brief treatment with 0.1 mM ATP, 10 mM MgCl\(_2\) in 0.1 M KCl, 50 mM Tris-HCl, pH 7.4, at 25 °C toward the latter stages of the purification. The concentration of the purified enzyme was determined spectrophotometrically at 280 nm (15). Enzymatic activity was assayed by the direct spectrophotometric method (16). Purity of the succinyl-CoA synthetase was confirmed by the appearance of a single band on standard polyacrylamide gels and of only the two characteristic subunit bands on gel electrophoresis under dissociating conditions with sodium dodecyl sulfate (1). All chemicals were of reagent grade or better and were used without further purification.

**Crystallization of Succinyl-CoA Synthetase**—The method used routinely to obtain large, single crystals has been microdialysis in 0.01 or 0.05 ml of protein solution contained by a semipermeable membrane in specifically designed Lucite "buttons" (17) was set to dialyze slowly against 3 ml of a precipitant solution. Succinyl-CoA synthetase, stored as a suspension in 65% (w/v) saturated ammonium sulfate solution, was collected by centrifugation. The precipitate was dissolved in a minimal volume of 50 mM Tris-HCl or 50 mM potassium phosphate, pH 7.4 (depending on the precipitant to be tried), and dialyzed extensively at 4 °C against several changes of the same buffer. Particular matter in the dialyzed sample was removed by high speed centrifugation. After measuring the protein concentration and specific enzymatic activity, the solution was dialyzed
RESULTS AND DISCUSSION

In an attempt to obtain large, single crystals of succinyl-CoA synthetase suitable for analysis by x-ray diffraction techniques, over 1000 crystallization trials have been conducted using various experimental conditions. These included combination and variation of protein concentration, pH (and therefore buffers), temperature, precipitants, and various additives. In all trials, only the phosphorylated form of the enzyme was used since this is known to be more stable than the dephosphorylated form (10). Crystals of succinyl-CoA synthetase have been obtained only with ammonium sulfate, potassium phosphate, or mixtures of sodium and potassium phosphate. From a phosphate medium at neutral pH, the enzyme crystallized as long needles, not at all suitable for x-ray analysis. With further experimentation, it was found that inclusion of small amounts (2–5% by volume) of acetone, 2-methyl-2,4-pentanediol, tert-butyl alcohol, or tert-amyl alcohol in the mother liquor produced a favorable change in the crystal habit. In phosphate media with addition of any of these solvents, succinyl-CoA synthetase crystallized as chunky plates or rectangular prisms. A micrograph of a representative crystal is shown in Fig. 1a where in this case 2-methyl-2,4-pentanediol was added to the precipitant solution after initial crystallization of needles had occurred. From a comparison of the structural formulae of the organic molecules as given in Table I (as well as from space-filling models), it is tempting to speculate that the dimethyl, hydroxy end of these small molecules is the common significant feature responsible for the reduction in the growth rate along the c axis of the crystals. The lack of success with other dissimilar organic solvents (e.g., methanol, ethanol, 1,2-ethanediol, 1,2-propanediol, or 2-butanol) under similar conditions would tend to rule out a simple change in the dielectric properties of the crystallization media.

Single rectangular prisms of succinyl-CoA synthetase were obtained most frequently from ammonium sulfate precipitant solutions at neutral pH when the additive was CoA (Fig. 1b).
Fig. 2. The effect of CoA on the solubility properties of succinyl-CoA synthetase in solutions of ammonium sulfate. Microdialysis was carried out at 21 °C using enzyme at a concentration of 11 mg/ml with a specific catalytic activity of 36 units/mg. In addition to the various concentrations of the primary and secondary precipitants tested, the dialysate contained 0.5 mM dithiothreitol, 0.1 mM EDTA, pH 7.3. The symbols represent the appearance of the protein solution in the button well and are as follows: ○, clear; ●, precipitate; □, rectangular prisms, a, without CoA added; b, with 0.5 mM CoA added.

Fig. 3. The minimum amount of CoA to effect crystallization of succinyl-CoA synthetase in solutions of ammonium sulfate. The experimental conditions and symbols are given in the legend to Fig. 2. The concentration of the secondary precipitant was constant at 100 mM. Using a value of $1.4 \times 10^8$ for the molecular weight of succinyl-CoA synthetase, the concentration of the enzyme was 0.08 mM.

TABLE II

| General conditions of crystallization | Radius of good reflections | Power of the x-ray generator | Time of exposure | Quality index ($/10^5$) |
|-------------------------------------|----------------------------|------------------------------|-----------------|--------------------------|
| (Na/K)PO$_4$ + acetone              | 0.04                       | 40/40                        | 1.4             | 0.3                      |
| (Na/K)PO$_4$ + TBA                   | 0.20                       | 40/40                        | 15              | 0.7                      |
| (Na/K)PO$_4$ + MPD                   | 0.30                       | 40/45                        | 20              | 1.0                      |
| (NH$_4$)$_2$SO$_4$ + CoA             | 0.16                       | 40/40                        | 15              | 0.4                      |
| (NH$_4$)$_2$SO$_4$ + CoA + anions*   | 0.46                       | 38/38                        | 16              | 5.8                      |
| (NH$_4$)$_2$SO$_4$ + CoA + MPD       | 0.05                       | 45/40                        | 2               | 0.3                      |

*a*, reciprocal lattice units; MPD, 2-methyl-2,4-pentanediol; TBA, tert-butyl alcohol.

In this case, phosphite plus arsenate.

Not surprisingly, a variation in the quality of x-ray diffraction was obtained from crystals grown under the different conditions. In general, diffraction was weak and fell off from the central region of the precession photographs, indicative of loose packing of the protein molecules or disorder in the crystals. All crystals had a limited lifetime in the x-ray beam, in the order of 24–48 h at a power of 40kV/40 mA. A comparison of the relative worth of diffraction from crystals grown under a variety of conditions is presented in Table II. The "quality index" ($Q$) has been developed to assess quantitatively the merit of one crystal over another with regard to the quality of diffraction. The index is numerically higher for crystals diffracting a larger area of measurable reflections for a lower power of x-rays and shorter time of exposure. The formula used to calculate this parameter is

$$Q = \frac{\pi(r/CF)^2}{i(kV - 9) \cdot t} \quad (2)$$

where $r$ is the radius of good reflections on a precession photograph, $CF$ the crystal to film distance, $i$ and $kV$ the operating current and voltage, respectively, of the x-ray generator, and $t$ the time of exposure. Table II shows the best results from crystals in a given group. The corresponding zero layer precession photographs obtained with crystals grown from a sodium/potassium phosphate mixture with addition of tert-butyl alcohol and from an ammonium sulfate medium plus CoA and arsenate ions are presented in Figs. 4 and 5, respectively. Similar diffraction patterns were observed within the groups of a given primary precipitant; that is, within errors of measurement, there were no changes in the pattern or spacing of reflections exclusive to either system within their respective additions. As can be seen from Table II, however, there was a great difference in the relative worth of the crystals with respect to the quality of diffraction. Crystals grown in media composed of ammonium sulfate plus CoA and anions displayed consistently the strongest diffraction with reflections extending to 3.4 Å. Within this group, the quality index varied from 0.7 for addition of 100 mM bicarbonate anions to 3.8 for the addition of 1 mM arsenate anions. The average value for the various anions tested was 1.7, indicating that the inclusion of these anions was beneficial to the growth of "good" crystals.

The limited diffraction obtained for crystals grown from a mixture of sodium and potassium phosphate plus organic...
solvents may have been a result of two factors complicating the mounting procedure: 1) these crystals floated in the mother liquor and 2) the organic solvents, particularly acetone and the alcohols, evaporated easily at room temperature. Attempts failed to replace the phosphate-organic solvent precipitant in these crystals with a sulfate/phosphate mixture or sulfate alone at a comparable ionic strength (other additions and conditions kept constant). Slow microdialysis of clear, birefringent crystals resulted in translucent ghosts or disintegration and subsequent precipitate formation. This would suggest that the molecular packing in the unit cell differed in the crystals from the two primary precipitant systems. The observation that the diffraction patterns (see for example Figs. 4 and 5a), as well as the physical properties of the two crystal types, are different corroborate this conclusion.

The preliminary crystallographic data from single crystals of succinyl-CoA synthetase are presented in Table III. The unit cell dimensions are average values of collective x-ray diffraction data. The a and b dimensions for crystals from sodium/potassium phosphate media are reliable to a relative standard deviation of 5%, the c dimension to only 10%. These poor measurements are a consequence of the limited diffraction displayed. The unit cell dimensions for crystals from ammonium sulfate media, reflecting larger Q values, are good, however, to a relative standard deviation of 1%. Both crystal types have large unit cell dimensions, particularly along the c axis of the tetragonal cell. Furthermore, the calculated values for Vm are at the higher end of the range given by Matthews (18). The lower protein fraction found (approximately one third of the unit cell) is consistent with the limited diffraction observed and the idea that proteins of higher molecular weight pack loosely to form crystals containing a relatively higher fractional volume of solvent (18). If there was a complete tetramer/asymmetric unit for the crystals grown from the phosphate media, the alternative values of Vm and protein fraction would be 1.96 \AA{}^3/dalton and 0.63, respectively. This result would not be consistent with the weak diffraction displayed; the quality indices are, at best, one quarter that observed with crystals from ammonium sulfate media. Moreover, the high protein fraction would be difficult to reconcile with the density properties of these crystals. It would appear, therefore, that the high phosphate concentration and/or the organic solvents "loosen" the tetramer such that a molecular 2-fold axis is generated (presumably between the \&dimers) and falls coincident with one of the 2-fold symmetry axes of P422. There is no question, however, that in the case of the crystals from ammonium sulfate media, there is one tetramer in the asymmetric unit. This is an important result because it supports the idea that the \&dimers of succinyl-CoA synthetase are arranged asymmetrically in the phosphorylated tetramer, as would be expected from the model of alternating sites cooperativity that we have developed for this enzyme (2, 11, 12, 19).

In preliminary examination of the precession photographs (Figs. 4 and 5), the h0l zone showed 4mm symmetry with no systematic absences evident along either axis and the h0l and 0kl zones showed 2mm symmetry with systematic absences only along the 00l layer line. For crystals from the phosphate media, reflections were observed at l = 2n, and for crystals from the sulfate media, reflections were observed at l = 4n. Hence, the probable space groups of these crystals are P422 and P422 (P422), respectively.

Future experimentation will be directed toward both crystal types. A detailed study of the effect of CoA, for example, may prove useful in procuring crystals that have shorter unit cell dimensions and/or diffract more strongly. At the present time, the crystals grown from the ammonium sulfate media are suitable for data collection using an oscillation camera with highly collimated x-ray beams, a reasonable start in the determination of the three-dimensional structure of succinyl-CoA synthetase.

**TABLE III**

| Crystal system | Crystals from (Na/K)PO\(_4\) | Crystals from (NH\(_4\))\(_2\)SO\(_4\) |
|----------------|-----------------------------|-------------------------------------|
| Unit cell dimensions | Tetragonal | Tetragonal |
| a = 94 Å | a = 99 Å |
| b = 94 Å | b = 99 Å |
| c = 248 Å | c = 399 Å |
| Intertaxial angles | α = β = γ = 90° | α = β = γ = 90° |
| Unit cell volume | 2.19 \times 10^6 Å\(^3\) | 3.91 \times 10^6 Å\(^3\) |
| Space group | P4\(_2\)22 | P4\(_2\)22 or P4\(_2\)22 |
| Molecules/unit cell | 8φ | 8φ |
| Vm \(^a\) | 3.91 Å\(^3\)/dalton | 3.49 Å\(^3\)/dalton |
| Crystal density | 1.19 gm/cm\(^3\) | 1.217 gm/cm\(^3\) |
| Molecules/asymmetric unit | 0.5 tetramer | 1 tetramer |

\(^a\) Using a value of 1.4 \times 10^6 for the molecular weight of succinyl-CoA synthetase.

**FIG. 4.** X-ray precession photograph of the h0l zone of a crystal of succinyl-CoA synthetase grown in phosphate medium with the addition of tert-butyl alcohol. The film was exposed for 20 h at a power setting of 40 kV/45 mA. In this case, \(\mu = 12.3\)°. Further details are given under "Experimental Procedures.”

**FIG. 5.** X-ray precession photographs of crystals of succinyl-CoA synthetase grown in ammonium sulfate media with the addition of CoA and arsenate anion. a, the h0l zone exposed for 16 h at 38 kV/38 mA; b, the h0l zone exposed for 20 h at 45 kV/42 mA. In both photographs, \(\mu = 13.5\)°.
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