# Folding and Assembly of the *Escherichia coli* Succinyl-CoA Synthetase Heterotetramer without Participation of Molecular Chaperones†

Guohua Fong‡ and William A. Bridger*
*Department of Biochemistry, University of Alberta Edmonton, Alberta, Canada T6G 2H7*
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ABSTRACT: Succinyl-CoA synthetase of *Escherichia coli* ($\alpha_2B_2$ subunit structure) has been shown to fold and assemble without participation by molecular chaperones. Renaturation experiments showed that purified bacterial chaperone GroEL has no effect on the folding and assembly of the active tetrameric enzyme. When isolated $^{35}S$-labeled $\alpha$ or $\beta$ subunits were incubated with GroEL in the absence of ATP, there was no complex formation between the subunits and GroEL. These in vitro results were confirmed by in vivo analysis of the folding and assembly of newly synthesized succinyl-CoA synthetase subunits. When expression of the subunits was induced in *E. coli* strains that bear GroEL or GroES temperature-sensitive mutations, the assembly of active succinyl-CoA synthetase was not affected as the temperature was raised to 43 °C. These and other observations are discussed that indicate that folding and assembly of succinyl-CoA synthetase may be independent of assistance by any chaperone.

It has long been recognized that the primary structure of proteins contains the information to direct their folding to produce the physiological functional tertiary structure and the formation of assemblies of subunits (Sela et al., 1957; Anfinsen et al., 1961; Stellwagen & Schachman, 1962). Recently, however, it has been found that the folding and assembly of some proteins in vivo can require the participation of molecular chaperones [for reviews, see Rothman (1989), Landry and Gierasch (1991), and Gething and Sambrook (1992)]. Most of these molecular chaperones are constitutively expressed heat shock proteins whose levels of expression often increase at higher temperatures. Proteins that are known to interact with chaperones fall into two major classes: those that are translocated through biological membranes and those that are assembled into complex multisubunit structures. For proteins destined for membrane translocation (secretion from prokaryotic cells, import into eukaryotic organelles, etc.), molecular chaperones such as *Escherichia coli* SecB (Collier et al., 1988; Watanabe & Blobel, 1989; Lecker et al., 1990) and yeast hsp70 cognates (Deshaies et al., 1988; Chirico et al., 1988) have been clearly shown to be required to maintain the protein in a conformation competent for translocation by preventing nonproductive interactions or assemblies. Many proteins, however, remain in the cytoplasm after their synthesis and fold and assemble into functional molecules or complexes. For some proteins that assemble to form multisubunit complexes, a product of the *groE* locus, GroEL [reviewed by Gething and Rothman (1992)], has been shown to participate, possibly by protecting against formation of nonproductive aggregates (Buchner et al., 1991). For folding of dihydrofolate reductase and rhodanese, it has been proposed that GroEL stabilizes the unfolded proteins in a disordered ("molten globule") state, which progresses to a more compact structure in the ATP-dependent process in the presence of GroES (Martin et al., 1991). We see a growing consensus that molecular chaperones are common participants in protein folding and assembly. In fact, such participation has been suggested to be a general requirement for the folding and assembly of intracellular, soluble proteins in vivo (Ellis & Hemmingsen, 1989; Martin et al., 1991). In variance with this proposed generalization, however, we report here that the folding and assembly of *E. coli* succinyl-CoA synthetase, a good example of this class of protein, occurs without effect of GroEL both in vitro and in vivo.

## Experimental Procedures

*Materials.* Succinyl-CoA synthetase of *E. coli* and its constituent $\alpha$ and $\beta$ subunits were prepared essentially as described previously (Wolodko et al., 1987), and the catalytic activity of this enzyme was measured by the direct spectrophotometric assay (Bridger et al., 1969). GroEL was purified essentially according to the procedure of Fayet et al. (1989). After the sucrose gradient centrifugation, further purification was achieved by gel filtration on a Sephacryl S-300 column. GroEL fractions were found to elute only slightly behind the void volume. This was consistent with the large size of the active form of GroEL composed of 14 subunits. The identity of the purified GroEL was further confirmed by SDS–polyacrylamide gel electrophoresis, which revealed a single band of 65 kDa, by the presence of an intrinsic ATPase with a specific activity as reported for the purified protein (Chandrasekhar et al., 1966), and by its ability to affect the refolding of denatured citrate synthase (Buchner et al., 1991). Purified GroEL was dissolved in 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 10% glycerol.

*Measurement of Folding and Assembly.* The in vitro folding and assembly of *E. coli* succinyl-CoA synthetase was carried out using modifications of procedures of Pearson and Bridger (1975), using denatured subunit preparation stocks prepared in 6 M deionized urea, 5% (v/v) acetic acid. The refolding buffer contained 50 mM potassium phosphate, pH 7.4, 10 mM $MgCl_2$, and 0.5 mM ATP (when present). To each milliliter of this solution was added 40 $\mu$L of buffer G (10% glycerol, 50 mM Tris-HCl, pH 7.5, 50 mM KCl), resulting in refolding buffer A. Alternatively, 40 $\mu$L of buffer G containing 2.65 mg/mL GroEL was added to 1 mL of the parental buffer to make refolding buffer B. The renaturation

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*To whom correspondence should be addressed.
‡Present address: Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada M5G 1X5.

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assays were performed as follows: 19.2 μL of α subunit (0.34 mg/mL) was mixed with 7 μL of β subunit (1.3 mg/mL), and the mixture was then neutralized with 26.2 μL of 0.80 N NaOH. A total of 10 μL of the neutralized subunits was diluted with 790 μL of refolding buffer A. The final SCS[1] concentration was 3.75 μg/mL, with a 1:1 molar ratio of α and β subunits. When included, GroEL was present at a ratio of one GroEL 14-mer for every SCS subunit. The reactions were incubated at 25 °C, and aliquots were withdrawn at various time intervals and assayed for SCS activity.

*Binding of GroEL to Succinyl-CoA Synthetase Subunits.* Radioactively labeled SCS was produced by inducing the expression at 43 °C in minimal medium supplemented with the following components: amino acids (all but methionine and cysteine), 0.06 mM each; 2 mCi of [$^{35}$S]methionine (Translabel, ICN) for 100 mL of culture; yeast nitrogen base to a final concentration of 0.34%. This medium was designed to allow both efficient protein synthesis and high specific radioactivity in protein. The labeled enzyme and subunits were purified according to previously reported procedures (Wolodko et al., 1981). The binding assay was performed as follows: 10 μL (~$10^6$ dpm) of purified SCS α subunit ($2.1 \times 10^6$ dpm/μg, 0.04 mg/mL in 6 M deionized urea, 5% acetic acid) was neutralized with 0.8 M NaOH, diluted with 0.5 mL of 50 mM potassium phosphate, pH 7.4, which contained 250 μg/mL GroEL. In the experiment described below, the molar ratio of SCS α subunit to GroEL complex was therefore about 1:5. As a control, phosphate buffer without GroEL was used to dilute the denatured subunit. The mixture was incubated at 25 °C for 20 min and then loaded onto a Sephacryl S-200 column (1 × 48 cm) equilibrated with the same phosphate buffer. Fractions of 0.65 mL were collected and counted.

*In Vivo Folding and Assembly of Succinyl-CoA Synthetase.* Measurement of the ability of GroEL or GroES to affect assembly of SCS in vivo was assessed as follows. *E. coli* strains used were NRK117 (*groEL44*, zje::Tn10), NRK233 (*groES619*, zje::Tn10), MC4100 (*groE*$^+$), CG2241 (*groEL44*), CG2244 (*groES619*), and B178 (Tilly & Georgopoulos, 1982). MC4100 and B178 are wild types with respect to the *groE* operon. All of the above strains were transformed with pGS202 (Buck & Guest, 1989) for high-level expression of succinyl-CoA synthetase under the control of the temperature-sensitive λ*cI*ts promoter. The transformed strains were grown in YT medium containing ampicillin to log phase at 30 °C and then shifted to 43 °C. One-milliliter aliquots were withdrawn at various time intervals, and these were immediately homogenized by sonication. After centrifugation, an aliquot from each supernatant was assayed for SCS activity.

## Results and Discussion

Succinyl-CoA synthetase from *E. coli* has an $\alpha_2\beta_2$ subunit structure (α = 29.6 kDa; β = 41.6 kDa). Previous work in this laboratory has established that purified subunits denatured in 6 M urea, 5% acetic acid can be renatured to form fully active enzyme (Pearson & Bridger, 1975; Wolodko et al., 1987). This by itself does not imply that the folding and assembly of SCS in vivo are independent of molecular chaperone catalysis. In fact, it has been shown that purified GroEL modulates the ability of pre-β-lactamase to refold in vitro, despite the fact that renaturation can also occur efficiently without GroEL (Laminet et al., 1990). We thus investigated how purified GroEL might affect the in vitro folding and assembly of SCS. Our results indicate that GroEL does not affect the reassembly of SCS in the presence or absence of ATP (Figure 1). These experiments were performed using relative concentrations of all proteins (SCS α and β subunits, GroEL complex) which were optimal for effects on in vitro assembly of citrate synthase (Bucher et al., 1991), but we cannot rule out the possibility that an in vitro GroEL effect might occur under conditions that we were not able to test.

It has been reported that GroEL and several other molecular chaperones form tight complexes with specific proteins or peptides in the absence of ATP (Lecker et al., 1989; Flynn

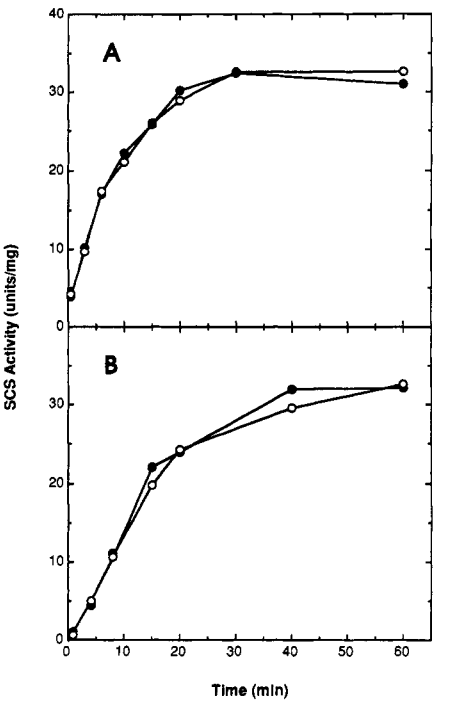


FIGURE 1: In vitro folding and assembly of *E. coli* succinyl-CoA synthetase. (A) Refolding and assembly in the presence of ATP: (○) refolding in the presence of purified GroEL; (●) refolding without added GroEL. (B) Refolding and assembly in the absence of ATP. All the assays were performed in the same manner as in (A), except that ATP and $MgCl_2$ were omitted from the refolding buffers, and the symbols are as in (A). Each data point is the average of triplicate renaturation experiments. See Experimental Procedures for details of the protocol.

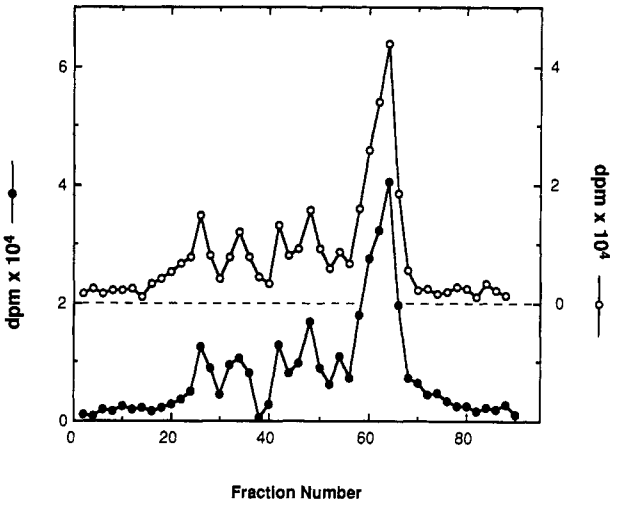


FIGURE 2: Assay for binding of GroEL to succinyl-CoA synthetase α subunit. $^{35}$S-Labeled α subunit was incubated with purified GroEL for 20 min at 25 °C (see Experimental Procedures), and the mixture was subjected to gel filtration on Sephacryl S-200: (○) with GroEL; (●) without GroEL. The data for the upper profile have been offset for clarity of presentation.

[1] Abbreviation: SCS, succinyl-CoA synthetase.

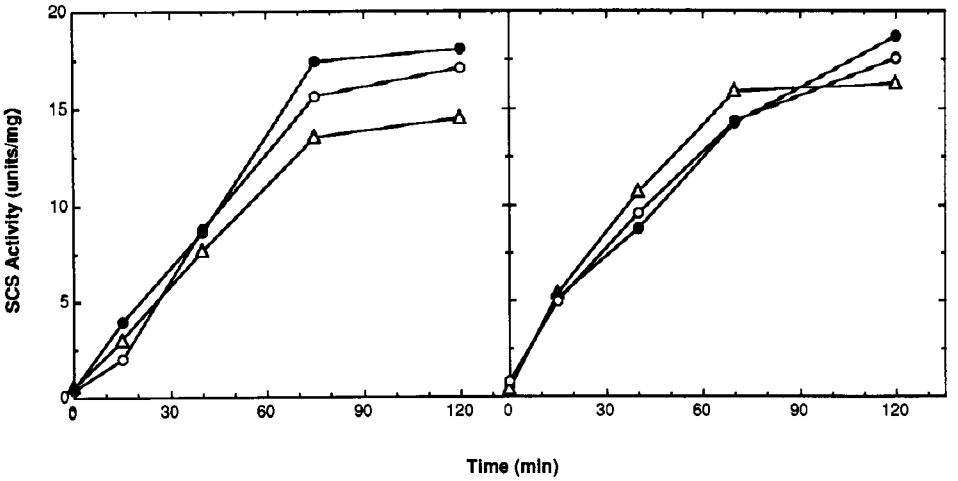


FIGURE 3: The in vivo assay of folding and assembly of succinyl-CoA synthetase in the absence of active GroEL or GroES. Strains bearing temperature-sensitive mutations in GroEL or GroES were transformed with pGS202 (see Experimental Procedures), and cultures were subjected to a shift in temperature from 30 to 43 °C at time zero. This treatment simultaneously induces overexpression of SCS $\alpha$ and $\beta$ subunits and inactivates GroEL and GroES in the temperature-sensitive mutants. The strains are as follows. Left: (△) NRK117 (GroEL$^{ts}$); (●) NRK223 (GroES$^{ts}$); (○) MC4100, parent nonmutated strain. Right: (△) CG2241 (GroEL$^{ts}$); (●) CG2244 (GroES$^{ts}$); (○) B178, parent nonmutated strain. Succinyl-CoA synthetase activities in each fraction were normalized to the protein concentration, and these are plotted versus the time following temperature shift.

et al., 1989). Consistent with this, GroEL inhibits in vitro folding of pre-$\beta$-lactamase in the absence of ATP but stimulates the folding reaction subsequent to ATP addition (Laminet et al., 1990). In contrast, the above results provide no suggestion of specific binding interactions between the SCS subunits and GroEL. To confirm this, we attempted to detect a complex formed between the $\alpha$ subunit and GroEL in the absence of $\beta$ subunit and ATP. $^{35}$S-Labeled SCS $\alpha$ subunit was incubated with GroEL (molar ratio $\alpha$:GroEL complex = 1:5) in the refolding buffer without ATP, and the sample was then analyzed by gel filtration on a Sephacryl S-200 column (Figure 2). Since GroEL forms tight complexes with its substrate polypeptide when ATP is not present, and since GroEL elutes as a very big particle in an S-300 gel filtration column, it was reasonable to expect a putative complex formed between $^{35}$S-labeled $\alpha$ subunit and GroEL to elute at, or just slightly behind, the void volume. The failure of GroEL to modify the elution profile of SCS $\alpha$ under these conditions is therefore taken as evidence against complex formation between the $\alpha$ subunit and GroEL, at least to the limit of detection in this experiment.[2] Considering the molar concentrations of the two proteins, we are confident that we would have detected a complex with $K_{diss} \leq \sim 10^{-6}$ M. These results are consistent with the failure of GroEL to affect assembly of SCS in vitro. They provide an important check on the in vitro assembly interpretation, because binding of GroEL is expected to occur over a wide range of concentrations in GroEL-assisted renaturation whether or not inhibition of renaturation is found.

An equivalent set of experiments was performed to attempt to detect formation of a complex between GroEL and the $\beta$ subunit. The results were the same: no change in the gel filtration profile of labeled $\beta$ was detectable in the presence of GroEL (data not shown).

We next wished to determine whether GroEL or GroES was required for folding and assembly in vivo. *E. coli* strains bearing temperature-sensitive mutations in the genes encoding GroEL or GroES (Tilly & Georgopoulos, 1982) were transformed with the plasmid pGS202, which allows the expression of succinyl-CoA synthetase under the control of tandem $\lambda$ promoters controlled by the thermosensitive repressor $\lambda cI857$ (Buck & Guest, 1989). When the mutant strains NRK117 and NRK233 (Kusukawa et al., 1989), or CG2241 and CG2244 (Tilly & Georgopoulos, 1982), are transformed with pGS202 and grown at 30 °C, expression of SCS is repressed but GroEL and GroES are active. Following a shift to 43 °C, $\lambda$ repressor is inactivated and thus transcription of the plasmid-encoded SCS genes occurs, and at the same time GroEL and GroES become inactive. The cultures continue to grow for a limited time after the temperature shift, since GroEL or GroES is not directly involved in processes related to cell division, such as DNA replication, transcription, and translation, but cell death eventually ensues because of the depletion of activities that need GroE for replenishment. Since GroEL and GroES are inactive at 43 °C, the expression of any significant amount of active SCS after the cultures have been shifted to the nonpermissive temperature may be interpreted as GroEL- or GroES-independent folding and assembly. We have examined the induction of SCS under these conditions and compared the results to the induction of SCS in the corresponding wild-type strains transformed with pGS202. The results (Figure 3) show no significant differences in the expression of SCS activity, whether or not active GroE proteins are present. This continued folding of SCS occurs under conditions where the mutant strains cease to carry out GroE-dependent phage head assembly and where $\beta$-lactamase secretion is blocked within 2 min of the temperature shift (Kusukawa et al., 1989). We conclude that GroEL and GroES are not involved in the folding and assembly of succinyl-CoA synthetase in *E. coli*.

Our demonstration of GroEL- and GroES-independent folding and assembly of SCS implies that the enzyme can fold and assemble in an uncatalyzed manner in vivo, although we are unable to exclude the possible involvement of other molecular chaperones. However, proteins such as SecB or trigger factor (Lill et al., 1988) are unlikely to catalyze the folding and assembly of SCS. These chaperones are specialized for facilitating bacterial protein export, and there is no suggestion

[2] It will be noted that the $\alpha$ subunit was eluted not as a single symmetrical peak, but with a few minor peaks eluting as larger particles. These larger species are not related to any possible interactions with GroEL, since they are seen whether or not GroEL is present. We attribute the presence of these peaks to the formation of aggregates, a process that we have observed repeatedly when the $\alpha$ subunit is dissolved in phosphate buffers.

that they may also be responsible for catalyzing the folding of intracellular proteins. Similarly, the *E. coli* heat shock protein DNaK has been implicated in protein export (Phillips & Silhavy, 1990) and in heat shock regulation (Bukau & Walker, 1990), but no independent role has been suggested for DnaK in assisting cytosolic protein folding and assembly. In contrast, GroEL and its eukaryotic homologues are thought to be involved mainly in facilitating intracellular assembly of multisubunit structures, although GroEL also functions in the bacterial protein export pathway (Lessing & Sambrook, 1992). A principal role for GroEL in assembly of cytosolic protein complexes would also be reasonable when one considers its abundance in *E. coli*. GroEL is maintained at a level of 2% of total proteins under normal growth conditions and increased to 12% when a culture is grown at 46 °C (Fayet et al., 1989). Genetic analysis has also demonstrated that the GroE operon is essential for normal cellular growth (Tilly & Georgopoulos, 1982), consistent with such a vital role. Two additional observations suggest that it is unlikely that folding and assembly of SCS may be catalyzed by other molecular chaperones. First, Wolodko et al. (1980) observed that in vitro folding and assembly of *E. coli* SCS was not affected, either positively or negatively, by the presence of *E. coli* cell extract from which small molecular weight species were removed. Second, we failed to observe any specific interaction between SCS subunits and cellular proteins when an $^{35}$S-labeled *E. coli* extract, depleted of ATP, was incubated with denatured SCS subunits under renaturation conditions (data not shown). The same result was obtained when the incubation was performed in the presence of ATP. Since the cell extract was highly labeled ($10^5$ dpm/$\mu$g of protein), and since a large excess of the cell extract was used (200 $\mu$g of extract for 1 $\mu$g of SCS), we expected to be able to co-precipitate any specifically bound proteins with anti-SCS antibodies. The failure to observe a co-precipitated radioactive protein thus implies that there is no tight interaction between SCS and any molecular chaperone present in the cell extract. Thus, all of our results suggest a chaperone-independent mechanism for intracellular folding and assembly of succinyl-CoA synthetase.

In this context, we note that the folding and assembly of the mammalian mitochondrial form of succinyl-CoA synthetase is much less facile than that of the *E. coli* enzyme that we report here. Refolding of the active $\alpha\beta$ dimer of pig heart SCS from its denatured subunits in vitro is inefficient and requires the addition of compounds such as poly(ethylene glycol) to the solvent (Nishimura et al., 1988). The route for synthesis and assembly of this enzyme in vivo is complex, involving synthesis in the cytosol, maintenance of translocatable conformation, mitochondrial import, and signal peptide removal prior to assembly in the mitochondrial matrix (Majumdar & Bridger, 1990). Many of the steps are likely to involve participation by molecular chaperones. In contrast, however, the subunits of *E. coli* SCS are synthesized in soluble, mature form in the cytosol where they assemble and function. Our results indicate that this can proceed without the participation of any known molecular chaperone.

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