The decarboxylase component (E1) of the human mitochondrial branched chain α-ketoacid dehydrogenase multienzyme complex (∼4–5 × 10^5 kDa) is a thiamine pyrophosphate-dependent enzyme, comprising two 45.5-kDa α subunits and two 37.8-kDa β subunits. In the present study, His₆-tagged E1 αβ₂ tetramers (171 kDa) denatured in 8 M urea were competently reconstituted in vitro at 23 °C with an absolute requirement for chaperonins GroEL/GroES and Mg-ATP. Unexpectedly, the kinetics for the recovery of E1 activity was very slow with a rate constant of 290 M⁻¹ s⁻¹. Renaturation of E1 with a similarly slow kinetics was also achieved using individual GroEL-α and GroEL-β complexes as combined substrates. However, the β subunit was markedly more prone to misfolding than the α in the absence of GroEL. The α subunit was released as soluble monomers from the GroEL-α complex alone in the presence of GroES and Mg-ATP. In contrast, the β subunit discharged from the GroEL-β complex readily rebounded to GroEL when the α subunit was absent. Analysis of the assembly state showed that the His₆-α and β subunits released from corresponding GroEL-polyypeptide complexes assembled into a highly structured but inactive 85.5-kDa αβ dimeric intermediate, which subsequently dimerized to produce the active αβ₂ tetramer. The purified αβ dimer isolated from Escherichia coli lysates was capable of binding to GroEL to produce a stable GroEL-αβ ternary complex. Incubation of this novel ternary complex with GroES and Mg-ATP resulted in recovery of E1 activity, which also followed slow kinetics with a rate constant of 138 M⁻¹ s⁻¹. Dimers were regenerated from the GroEL-αβ complex, but they needed to interact with GroEL/GroES again, thereby perpetuating the cycle until the conversion from dimers to tetramers was complete. Our study describes an obligatory role of chaperonins in priming the dimeric intermediate for subsequent tetrameric assembly, which is a slow step in the reconstitution of E1 αβ₂ tetramers.

Molecular chaperonins are generally protein agents that promote proper folding of other polypeptides in an energy-depend-
is still a matter of debate, there is evidence to suggest that this species represents an intermediate in the protein folding cycle (12, 14). During the cycle, it is believed that only one of the two GroEL cavities or rings accommodates the unfolded polypeptide. However, recent electron micrograph data indicate that both cavities of GroEL harbor unfolded polypeptides (15, 16). Correlations of the symmetric GroEL4-(GroES)2 complex with the protein folding activity of chaperonins have been shown (16, 17). However, the mechanism of chaperoning action for this putative symmetric hetero-oligomer is not clear.

Despite the advances described above, only limited information is available concerning the role of chaperones during the assembly of oligomeric proteins. Earlier studies with mitochondrial ornithine transcarboxylase (18), glutamate synthetase (19), and malate dehydrogenase (20) suggest that chaperonins GroEL and GroES mediate the folding of assembly competent monomers, followed by spontaneous assembly of monomers into active homo-oligomers. In the case of heterodimeric proteins, for example, the native αβ dimer of bacterial luciferase, a prevailing model is that GroEL binds separately to the folding intermediate of either subunit and the folded α and β subunits released in the presence of GroES and Mg-ATP assemble to form the active enzyme (21). In a recent study, it was shown that GroEL/GroES modulate the kinetic partitioning of the β subunit intermediate of bacterial luciferase between two alternate pathways to increase the yield of native αβ dimers (22). As for Bacillus pyruvate dehydrogenase, the α and β subunits individually expressed in Escherichia coli, when incubated in vitro, spontaneously assemble into the native αβ2 structure (23).

We are interested in understanding the chaperonin-mediated biogenesis of mitochondrial macromolecular structures using the human branched chain α-ketoadehydrogenase (BCKD) complex as a model system. The mitochondrial BCKD complex (~5 × 105 kDa) is organized around a cubic core comprising 24 dihydrolipoyl acyltransferase (E2), to which branched chain α-ketoadehydrogenase (E1), dihydrolipoyl dehydrogenase (E3), a specific kinase, and a specific phosphatase are attached through ionic interactions (24). We reported earlier that GroEL/GroES are essential for in vitro reconstitution of the 24-meric inner core of the E2 component (25). The E1 component is a thiamine pyrophosphate (TPP)-dependent enzyme, which consists of two 45.5-kDa α subunits and two 37.8-kDa β subunits. We have shown previously that co-transformation of GroEL/GroES into E. coli expressing mammalian E1 subunits resulted in an over 500-fold increase in the yield of the active E1 αβ2 tetramer, compared with the single transformant without overexpression of chaperonins (26, 27). However, the precise steps in chaperonin-mediated assembly pathway of E1 remain to be elucidated.

In this paper, we report in vitro reconstitution of urea-denatured human E1, which shows an absolute requirement for GroEL/GroES and Mg-ATP. Surprisingly, the kinetics of E1 reconstitution is markedly slower than that of other proteins, for example, mitochondrial malate dehydrogenase refolded under similar conditions. Moreover, a novel ternary complex resulting from the interaction of GroEL with a large (85.5 kDa) αβ dimeric intermediate during assembly is described. We show that the chaperonin-dependent conversion of inactive dimers to active tetramers is a slow step in E1 assembly. These findings provide evidence for an obligatory role of chaperonins in priming the assembly intermediate for subsequent higher order oligomerization.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—**E. coli CG-712 cells (an ES86 strain) and the pGroESL plasmid overexpressing GroEL and GroES were kind gifts of Drs. George Lorimer and Anthony Gatenby of the DuPont Experimental Station (Wilmington, DE). The pTrcHisB plasmid vector was obtained from Invitrogen (Carlsbad, CA). The pHisT-hE1 plasmid for co-expression of Hisαα and untagged β subunits of human E1 was described previously (28). The Hisαα subunit contained a His tag and the tobacco etch virus protease cleavable linker fused to the N terminus of the α subunit.

**Expression and Purification of GroEL and GroES—**The pGroESL plasmid was transformed into E8-6 CG-712 cells, which were grown at 37 °C under chloramphenicol selection to A600 of 0.6. The expression of GroEL and GroES was induced by IPTG overnight at 37 °C. Cell lysates were prepared by sonication in a lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine as described previously (28). GroEL was purified from the lysates according to the method described by Clark et al. (29) that included a critical Reactive Red column step with the following modifications. Prior to DEAE-Sepharose column fractionation, the protein sample was treated with 10 mM Mg-ATP at 37 °C for 2 h. The collected GroEL fractions from the ion exchange column were concentrated and treated with 10 mM Mg-ATP again at 37 °C for 2 h, followed by purification on Sephacyr-S-400 HP column with Mg-ATP omitted from the column buffer. Eluted GroEL fractions were pooled, concentrated, and purified on a Reactive Red column. In a concurrent purification scheme, fractions from the above DEAE-Sepharose column containing GroES were pooled, and GroES was purified by passage over a Reactive Red column as described previously (30). Concentrations of GroEL and GroES were determined spectrophotometrically using published extinction coefficients of 1.22 × 104 M−1 cm−1 (30) and 1.2 × 104 M−1 cm−1 (31), respectively.

**Expression and Purification of Recombinant Human E1 Proteins—**CG-712 cells co-transformed with pGroESL and pHisT-hE1 plasmids were grown in YTOK (32) medium. Expression of Hisαα and untagged β subunits and GroEL/GroES was induced by IPTG. Assembled human Hisαα-E1 was isolated from cell lysates and purified by Ni-NTA (Qiagen, Chatsworth, CA) column chromatography as described previously (28). To remove the Hisαα tag, the purified fusion human E1 was digested with the tobacco etch virus protease at 4 °C overnight. The released Hisαα tag was removed by Ni-NTA extraction.

**Denaturation and Reconstitution of Human E1 αβ2 Tetramers—**Purified Hisαα-tagged or untagged human E1 was denatured for 1 h at 23 °C in a denaturing buffer containing 8 M urea, 50 mM potassium Pi, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 0.1% Tween 20, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride at a final E1 tetramer concentration of 12.5 μM. Denatured E1 was quickly diluted 100-fold on a Vortex mixer into a refolding buffer consisting of 50 mM potassium Pi, pH 7.5, and 0.5 mM DTT. Aliquots were taken to which CDTA was added to a final concentration of 0.5 mM. The refolding reaction was initiated by addition of Mg-ATP to a final concentration of 10 mM. The reaction mixture was incubated at 37 °C for up to 30 h. At indicated times, aliquots were taken to which CDTA was added to a final concentration of 25 mM to discontinue the folding reaction. Samples were kept at −80 °C until analysis. A control, purified human E1 without urea treatment was incubated in the refolding buffer under the same conditions. For samples to be extracted with Ni-NTA resin, 1 mM DTT was replaced with 20 mM β-mercaptoethanol in the refolding buffer.

**Assays for E1 Activity in a Reconstituted BCKD System—**The assay mixture contained 100 mM potassium Pi, pH 7.5, 2.5 mM NAD1, 2 mM MgCl2, 100 mM NaCl, 0.6 mM CoA, 0.2 mM TPP, 0.1% Triton X-100, 7-nM recombinant bovine E2, and 0.4 μM recombinant human E3. To 0.3 ml of the assay mixture, 20 μl of the aliquot from the refolding reaction was added. The enzyme reaction of the BCKD complex was initiated by addition of 10 μl of 6.4 mM (final concentration, 0.2 mM) α-keto[1-14C]isovalerate (specific radioactivity, 1,500–1,800 cpm/nmol). After incubation at 37 °C for 20 min, the reaction was quenched by addition to a final concentration of 6% trichloroacetic acid. Released radiolabeled CO2 was trapped in a paper wick soaked with 1 N NaOH and measured in a Beckman scintillation counter. The assay was linear for up to 1 μM of the purified human E1 protein. The rate of decarboxylation with α-keto[1-14C]isovalerate as substrate by E1 in the reconstituted BCKD system was 15-fold higher than that by E1 alone (33). Assay components in the BCKD system had no measurable effects on E1 denaturation (data not shown).

**Preparation of Individual GroEL-Hisαα and GroEL-β Complexes—**Hisαα-tagged human E1 was denatured in the above denaturing buffer containing 10 mM β-mercaptoethanol. The Hisαα subunit was extracted with Ni-NTA resin pre-equilibrated in the same denaturing buffer. The untagged β subunit remained in the supernatant. The Hisαα subunit was eluted from Ni-NTA resin with 250 mM imidazole in the denaturing...
buffer. Separated individual subunits were concentrated in Centri-
con-30 concentrators and rapidly diluted into the refolding buffer con-
taining various stoichiometric amounts of GroEL. The GroEL-polypep-
tide complexes were purified on a 10–30% sucrose density gradient in
Beckman SW41 rotor at 210,000 g for 18 h at 4 °C. Fractions con-
taining the GroEL-GroES-α or the GroEL-β complex were pooled, concen-
trated, and stored at −80 °C. The stoichiometries of GroEL-polypeptide
complexes were determined by densitometry scanning of Coomassie
Blue-stained SDS-PAGE gels. GroEL, His₆-α, and untagged β mono-
ers that were used as standards and run on the same gel were linear
up to 30, 5.5, and 4.5 μg, respectively. The amount of sample to be
determined was adjusted to be within the linear range.

Expression and Purification of the αβ Dimeric Intermediate—CG-712
cells co-transformed with pHIsT-hE1 and pGroESL plasmids were
grown at 37 °C in the C2 broth minimal medium and induced for
expression of E1 subunits with IPTG for 3 h. The C2 broth minimal
medium was modified from the C broth medium with low sulfate and
amino acid contents as described previously (34). The growth medium
was concentrated by centrifugation in Millipore Ultra free-15 filter con-
centrators and rapidly diluted into the refolding buffer containing
8 M urea. Denatured E1 was rapidly diluted 100-fold into a refolding buffer
at pH 7.5 in the presence or absence of 2 μM GroEL and 4 μM GroES. The refolding reaction was initiated by addi-
tion of Mg-ATP to a final concentration of 10 mM and incubated at
23 °C. At indicated time points, aliquots were taken, and the reaction
was terminated by addition of CDTA to 25 mM. Samples were assayed
for the reconstituted BCKD activity in the presence of excess in vitro
lipoylated recombinant E2 and recombinant E3 with α-keto[1-³¹C]
isovalerate (1, 800 cpm/nmol) as a substrate (see “Experimental Proce-
dures”). The 100% control activity represents the activity of the same
amount of E1 without denaturation at 2.08 μmol CO₂ released/min/mg
E1. ●, GroEL/GroES and Mg-ATP; ○, GroEL/GroES; △, no addition.

RESULTS

Reactivation of Urea-denatured His₆-tagged Human E1—
Recombinant His₆-tagged E1 was denatured in a buffer contain-
ing 8 M urea. The denatured E1 tetramer was rapidly diluted 100-fold into a refolding buffer contain-
ing various stoichiometric amounts of GroEL and GroES, respectively, and 1 mM DTT. Fig. 1 shows that the time course for the recovery of E1 activity follows strikingly slow kinetics during the 30-h incubation at 23 °C with a second order rate constant of 290
m⁻¹ s⁻¹. With the same GroEL/GroES preparations, refolding of urea-denatured mitochondrial malate dehydrogenase reached a plateau of 80% recovered activity in 40 min with a second order rate constant of 1.2 × 10⁴ m⁻¹ s⁻¹, similar to that
reported previously (20). The reconstitution of His₆-tagged E1 was
completely dependent on GroEL/GroES and Mg-ATP. The 100% control E1 activity (2.08 μmol/min/mg E1 protein) as-
sayed in the presence of E2 and E3 was comparable with that
reported previously (35). No E1 activity was reconstituted when
Mg-ATP or GroEL/GroES and Mg-ATP were omitted from the refolding mixture (Fig. 1). When urea-denatured un-
tagged E1 was used as a substrate, its reactivation kinetics was
very similar to that obtained with urea-denatured His₆-tagged
E1 (data not shown).

Differential Dependence of E1 Subunits on GroEL for E1
Reconstitution—The requirement for chaperons for reconsti-
tution of E1 activity was demonstrated by experiments in
which GroEL addition to the folding reaction mixture was
delayed. The His₆-α subunit denatured in 8 M urea was rapidly
diluted into the refolding buffer at 23 °C without chaperonins. At
different times, aliquots were taken, and a 4-fold molar excess of the GroEL/GroES mixture (molar ratio 1:2) relative to
the His₆-α monomers was added. The mixture was then combined
with an 1:1 stoichiometric amount of the GroEL-β complex with
respect to the His₆-α subunit to reconstitute E1 activity. E1 activity regained with GroEL-His₆-α (no delay in GroEL addi-
tion) and GroEL-β as substrates was 100% (Fig. 2, 0 min). In a
converse experiment, GroEL was added to the denatured un-
tagged β subunit in a time-dependent fashion, and stoichiomet-
ric amounts of the GroEL-His₆-α complex were added to reconsti-
tute E1 activity. Delayed additions of GroEL to denatured
His₆-α caused a sharp concentration-dependent decline in the
ability of the latter to regain E1 activity with the GroEL-β complex (Fig. 2). The second order rate constant for the reduc-
tion in regained E1 activity resulting from delayed addition of
GroEL to the denatured His₆-α subunit was 2.75 × 10⁴ M⁻¹ s⁻¹
at 23 °C. The delayed addition of GroEL to denatured β re-
sulted in a more precipitous fall in renatured E1 activity with
a second order rate constant of 9.79 × 10⁴ M⁻¹ s⁻¹, which was
3.5-fold higher than that obtained with the denatured His₆-α.
Without added GroEL, the denatured His₆-α and β subunits
rapidly aggregated upon dilution of the denaturant, as meas-
ured by light scattering at 488 nm (data not shown).

Effects of Polypeptide/GroEL Stoichiometry on Reconsti-
tution of E1 Activity—To further investigate differential depend-
ence on GroEL/GroES between the α and β subunits for proper
folding, different molar ratios of the polypeptide-GroEL (abbrevi-
ated as EL) complex were used in the refolding studies. To
produce complexes with different (subunit):EL molar ratios, stoichio-
metry, increasing amounts of the denatured His₆-α or β subunit in
8 M urea were diluted at 4 °C into the refolding buffer contain-
ing GroEL. After purification on a sucrose density gra-

Fig. 1. GroEL/GroES-dependent reconstitution of urea-denatured
human E1. Recombinant His₆-tagged E1 (12.5 μM, tetramer), with a His₆ tag and a tobacco etch virus protease-cleavable linker fused
to the N terminus of the α subunit, was denatured in a refolding buffer containing 8 M urea. Denatured E1 was rapidly diluted 100-fold into a refolding buffer at pH 7.5 in the presence or absence of 2 μM GroEL and 4 μM GroES. The refolding reaction was initiated by addition of Mg-ATP to a final concentration of 10 mM and incubated at
23 °C. At indicated time points, aliquots were taken, and the reaction
was terminated by addition of CDTA to 25 mM. Samples were assayed
for the reconstituted BCKD activity in the presence of excess in vitro
lipoylated recombinant E2 and recombinant E3 with α-keto[1-³¹C]
isovalerate (1, 800 cpm/nmol) as a substrate (see “Experimental Proce-
dures”). The 100% control activity represents the activity of the same
amount of E1 without denaturation at 2.08 μmol CO₂ released/min/mg
E1. ●, GroEL/GroES and Mg-ATP; ○, GroEL/GroES; △, no addition.
In Experiment 2, the unfolded preformed GroEL-subunit complexes, without elapsed times, is 100%. The His6-1:2 at a 4-fold molar excess were added to different concentrations of chaperonins. At the indicated elapsed time, GroEL/GroES (molar ratio, the (EL)14 complex was added to different concentrations of denatured β subunit preincubated in the refolding buffer at different elapsed times. The preformed GroEL-His6-α complex was added, and refolding was initiated by the addition of 5 mM Mg-ATP. The concentrations of the denatured His6-α subunit were 0.08 μM (●) and 0.16 μM (○). The concentrations of the denatured β subunit were 0.125 μM (▲) and 0.25 μM (△). Reconstituted E1 activity obtained with both preformed GroEL-subunit complexes, without elapsed times, is 100%.

As shown in Fig. 4A, a significant portion of the His6-α subunit was released from the GroEL-His6-α complex. The discharged His6-α subunit was present as soluble monomers as determined with a calibrated FPLC gel filtration column (see Fig. 7). A small fraction of GroEL monomers resulting from centrifugation in the presence of Mg-ATP (36) co-sedimented, but were not complexed, with His6-α monomers. In contrast, when the GroEL-β complex was incubated with GroES and Mg-ATP, no free soluble β protein was detected on the sucrose density gradient (Fig. 4B). To ask whether the β subunit was capable of being released from the GroEL-β complex alone, the C-terminally tagged His6-β subunit was used to form the GroEL-His6-β complex. When Ni-NTA was present in the refolding mixture containing GroEL-His6-β, GroES, and Mg-ATP, the His6-β subunit was extracted (data not shown). This indicates that the β subunit is indeed released from the GroEL-β complex transiently but cannot exist as a soluble species without assembly with the α and rebinds to GroEL.

**Assembly State of E1 Folding Intermediates**—The complete refolding mixture with urea-denatured His6-tagged E1 as a substrate was sampled at different times during a 24-h incubation at 23 °C. Aliquots were treated with Ni-NTA resin, and bound proteins were eluted with a buffer containing 250 mM imidazole. Sucrose density gradient centrifugation was routinely used to separate protein species in the refolding mixture because: 1) It was capable of handling multiple samples with relatively large volumes (0.5–1 ml), which normally resulted after diluting out the denaturant for refolding. Sample concentration required for gel filtration often led to the precipitation of assembly intermediates (see Fig. 5). 2) The method caused a minimal dilution of samples, which was usually associated with gel filtration as a result of diffusion. 3) Folded or assembled E1 species were stable on the sucrose density gradient, and their identities were confirmed by calibrated FPLC gel filtration (see Fig. 7).

As shown in Fig. 5, at zero time, a detectable amount of aggregated His6-α subunits and a small fraction of the GroEL-His6-α complex extractable with Ni-NTA resin sedimented to near the bottom of the sucrose density gradient. At 45 min into the refolding reaction, small amounts of putative species of His6-α monomers (fractions 4 and 5), His6-tagged αβ dimers (fractions 4–6), and αβ2 tetramers (fractions 7–10) were present in the gradient. Because His6-α monomers and His6-tagged αβ dimers partially overlapped on the sucrose density gradient, the appearance of the untagged β in the Ni-NTA extract served as a true indicator for the assembly state of folding intermediates. At the 2-h time point, an appreciable amount of the β subunit was present in fractions 4–8. However, unassembled His6-α monomers still persisted, resulting in significantly higher abundance of the α subunit than β in dimer fractions 4–6. The data suggest that the assembly of the β subunit with the α is rate-limiting in the formation of αβ dimers. During the prolonged incubation (6–24 h), there was not only more Ni-NTA extractable β present but also a significant shift in the position of the β subunit from that of dimers to tetramers (fractions 7–9). The data depict a slow but ostensible conversion from putative dimers to tetramers. Enzyme assays indicated a close correlation between the recovery of E1 activity and the amount of tetramers formed during the refolding (data not shown). The apparent substoichiometric amounts of the β subunit relative to the α in assembled dimers and tetramers resulted from the poorer binding of the β subunit to Coomassie Blue than the α (molar dye intensity β:α = 1:1:4).

**Expression and Purification of the Dimeric Assembly Intermediate**—To produce a large amount of αβ dimers for charac-
The α or β monomer complexed with the GroEL 14-mer at different stoichiometries (right panel) was prepared as described under “Experimental Procedures.” The molar ratio of the monomer (α or β subunit) to the 14-mer (GroEL) was determined by SDS-PAGE and densitometry scanning of the Coomassie Blue-stained protein bands. Actual molar concentrations (in μM) of each stoichiometric complex (right panel) were as follows: (α)1:(EL)14 = 1:2 (0.58:1.2); (β)1:(EL)14 = 2:1 (0.74:0.35); (β)1:(EL)14 = 2:1 (1.48:0.63). The concentrations of urea-denatured α or β subunit were both at 1 μM. To carry out the refolding reaction in Experiments 1–4, 10 μM GroES and 1 mM Mg-ATP were added to each refolding mixture. Refolding was allowed to proceed at 23 °C for 16 h. E1 activity (left panel) was assayed and expressed as radioactivity (in cpm) of 14CO2 released from substrate α-keto[1-14C] isovalerate in 20 min at 37 °C.

The combined αβ dimer fractions from the sucrose density gradient was further purified by FPLC gel filtration. Purified Hisα-tagged tetramers, Hisβ-tagged αβ dimers, and Hisα-α monomer were eluted from a calibrated HiLoad Superdex 200 column at peak fractions 39, 42, and 46, respectively (Fig. 7). The elution profiles confirmed the identities of folded or assembled E1 species separated on sucrose density gradients (Figs. 4–6). The FPLC-purified αβ dimer was free of contamination by Hisα-α monomers and assumed a native-like structure as indicated by its tryptophane fluorescence at 345 nm, similar to the αββ αββ tetramer (data not shown).

Binding of αβ Dimers to GroEL—In our earlier studies, when Hisα-tagged E1 carrying a T265R human mutation was expressed in the ES30 CG-712 host co-transformed with the pGroESL plasmid, a ternary complex comprising GroEL, the mutant Hisα-α, and untagged β subunits was isolated from the bacterial lysate by Ni-NTA extraction (data not shown). The accumulation of the αβ dimer in E1 assembly strongly suggested that the GroEL-αβ ternary complex may have resulted from binding of the assembly intermediate to GroEL. To address this question, the FPLC-purified Hisα-αβ dimer (5 μM) from E. coli lysates (Fig. 7B) was incubated with GroEL (5 μM) for 4 h at 23 °C. The incubation mixture was then separated by HPLC gel filtration. The αβ dimer and GroEL were co-eluted as a large single peak at 6.5 min (Fig. 8, peak 1). SDS-PAGE analysis showed the GroEL-αβ ternary complex with a 1:1 stoichiometry (Fig. 8, inset). The excess unbound αβ dimer was eluted as a minor peak at 8.9 min (Fig. 8, peak 2). Incubation of the Hisα-tagged αββ tetramer with GroEL did not form a complex as determined by sucrose density gradient centrifugation (data not shown).

Slow Reconstitution of Active αββ Tetramers with the GroEL-αβ Complex, GroES, and Mg-ATP—The isolation of the stable GroEL-αβ complex prompted us to ask whether this ternary complex was an obligatory intermediate in the E1 assembly pathway and whether GroES was essential for the conversion of dimers into tetramers. The purified GroEL-αβ complex (Fig. 8) was incubated with GroES and 5 mM Mg-ATP at 23 °C. Aliquots collected at different times were assayed for E1 activity. Fig. 9 shows that E1 activity was reconstituted from the GroEL-αβ complex, but again with very slow kinetics with a second order rate constant of 138 M$^{-1}$ s$^{-1}$. The recon-
GroEL/GroES-dependent αβ2 Reconstitution

FIG. 5. Formation of the αβ dimeric intermediate and αβ2 tetramers as analyzed by sucrose density gradient centrifugation. His6-α human E1 (12.5 μM of tetramers) denatured in 8 M urea was diluted 100-fold into the refolding buffer containing 1 μM GroEL and 4 μM GroES. The refolding reaction at 23 °C was started by additions of Mg-ATP (final concentration, 5 mM). At each time point, an aliquot was taken and kept on ice. A portion was used for enzyme assays, and the remainder was extracted with Ni-NTA resin. After washing for three times with the refolding buffer (without GroEL/GroES and Mg-ATP), bound protein species were eluted with the same buffer containing 250 mM imidazole. The eluates were separated on a 10–30% sucrose density gradient spun at 210,000 × g for 18 h. Fractionated samples were precipitated with 6% trichloroacetic acid and separated on 12% SDS gels, followed by staining with Coomassie Blue. The molecular mass markers used for calibrations (in kDa) were: bacterial lipoylated enzyme LplA (35), bovine serum albumin (60), human E3 (110), maltose-binding protein-human BCKD kinase (340), and GroEL (840).

The assembly state of E1 subunits released from the GroEL-αβ complex was also studied. The sucrose density gradient profile showed that the dimer released from GroEL slowly converted to the tetramer during a 18-h incubation at 23 °C, similar to that observed with the urea-denatured E1. The Ni-NTA-extracted refolding mixture taken at the 2-h time point consisted primarily of the αβ dimer and a trace amount of the αβ2 tetramer as separated on the sucrose density gradient. Incubation of this refolding mixture without chaperonins for 16 h at 23 °C did not cause the conversion of the dimer to tetramer (Fig. 10A). However, a similar incubation with GroEL/ES and Mg-ATP resulted in a complete conversion of αβ dimers to αβ2 tetramers (Fig. 10B).

Most investigations into chaperonin-mediated protein folding to date have focused on small monomeric proteins or synthetic polypeptides (1, 2). Although these studies have provided significant insights regarding the mechanisms of GroEL/GroES action, relatively little is known in the area of chaperonin-dependent folding and assembly of large hetero-subunit proteins. To address this problem, we have chosen the E1 component of the human mitochondrial BCKD complex as a model system. Here, we show that the reconstitution of the αβ2 tetramer of E1 is absolutely dependent on GroEL/GroES. In contrast, other proteins such as rhodanese (36, 37), bacterial luciferase (22), and mitochondrial malate dehydrogenase (30) can either spontaneously refold or be assisted by the chaperonins during folding. Both the denatured α and β subunits depend on GroEL/GroES for their ability to reconstitute E1 activity. The system thus allows for an elucidation of how these chaperonins mediate the cross-talk between the α and the β subunits during E1 αβ2 assembly. Moreover, the BCKD complex is deficient in patients with heritable maple syrup urine disease (MSUD), resulting in severe ketoacidosis, neurological derangements, and mental retardation (38). We have shown previously that a subset of MSUD mutations in the α subunit impair E1 assembly in vivo (39, 40) and in E. coli (28). The availability of the reconstituted E1 system will facilitate studies on the mechanism by which MSUD mutations perturb the normal interaction between chaperonins and E1 subunits.

A striking feature of chaperonin-dependent E1 reconstitution is the slow kinetics. The rate constant of 290 M⁻¹ s⁻¹ is 2 orders of magnitude slower than that for the refolding of mitochondrial malate dehydrogenase (Ref. 20 and this study). The slow rate of folding and assembly for the E1 tetramer in vitro is consistent with the sluggish expression of recombinant E1 in E. coli. In the latter experiment, the levels of the assembled recombinant His6-α and untagged β subunits reached a plateau after induction with IPTG for 7 h at 37 °C; however, the maximal E1 activity was not observed until 12 h after the induction (data not shown). The slow pace of E1 biosynthesis also coincides with the relatively low turnover rate of the enzyme components of mammalian mitochondrial α-ketoacid dehydrogenase complexes. In a previous study, half-lives for the α and β subunits of mitochondrial pyruvate dehydrogenase were found to be identical at 41 h in murine 3T3-L1 preadipocytes and 49 h in differentiated adipocytes (41). At present, however, we cannot rule out the involvement of additional chaperones or assembly factors that might enhance the rate of E1 reconstitution in vitro. For example, COX14 (42) and COX15 (43) are mitochondrial membrane proteins essential for assembly of yeast
cytochrome oxidase. Peptidyl prolyl isomerase was shown to accelerate the reactivation of the antibody Fab fragment (44), but our preliminary data to date indicate that this enzyme/chaperone has no effect on E1 assembly either in the presence or absence of GroEL/GroES. It is also plausible that the slow kinetics of E1 refolding may be due to the absence of an additional mitochondrial chaperone Hsp70 in the refolding mixture. The mitochondrial Hsp70, upon anchoring to matrix TIM44, is proposed to function as an ATP-driven import motor that binds to the incoming precursor and causes unfolding and inward translocation of the protein (45, 46). Successive action of DnaK (a bacterial Hsp70 homologue), DnaJ, and GroEL has been shown to augment the efficiency of in vitro refolding through stabilization of folding intermediates (47).

The reconstitution of E1 activity from individual GroEL-α and GroEL-β complexes permitted studies on the differential dependence of the two distinct E1 subunits on GroEL for folding. The more stringent requirement of the β subunit than the α for the chaperonin was indicated by: 1) the more rapid loss of regained E1 activity when the addition of GroEL to the denatured β subunit was delayed and 2) the inability of the β subunit to reconstitute E1 activity with the α2EL14 = 2:1 complex, in which most, if not all, GroEL cavities were initially occupied by the α subunit (Fig. 3). In this context, it is significant that in the absence of the folded α subunit, the β released from the GroEL-β complex rebound to GroEL, resulting in no net discharge of the β subunit from the chaperonin (Fig. 4B). This result explains our earlier observation that the bovine β subunit overexpressed alone in E. coli was largely insoluble and a fraction of the recombinant subunit formed a stable

![Image](https://example.com/image.png)

**FIG. 6.** Accumulation of inactive αβ dimers in E. coli grown in C2 broth minimal medium. ES*CG-712 cells were co-transformed with the pGroESL plasmid and the pHisT-hE1 plasmid carrying the Hisα-α and the untagged β cDNAs. Cells grown in the C2 broth minimal medium were induced for expression of E1 subunits with IPTG for 3 h. Cell lysates were extracted with Ni-NTA resin. After washing the resin with a phosphate buffer at pH 7.5 containing 500 mM KCl and 15 mM imidazole, Hisα and associated untagged β subunits were eluted with 250 mM imidazole in the same buffer. The concentrated eluate was subjected to sucrose density gradient centrifugation, and the fractions were analyzed by SDS-PAGE (upper panel). E1 activity in eluted fractions was assayed (lower panel). One m-unit is equivalent to 1 nmol CO2 released/min. The molecular mass markers used for calibration (in kDa) were: ovalbumin (44), bovine serum albumin (67), human E3 (110), aldolase (158), and catalase (232).

![Image](https://example.com/image.png)

**FIG. 7.** FPLC gel filtration profiles of αβ, tetramers (A), αβ dimers (B), and α monomers (C). Hisα-tagged αβ, tetramers, αβ dimers were expressed in E. coli and partially purified by Ni-NTA extraction and sucrose density gradient centrifugation. The concentrated eluate was subjected to sucrose density gradient centrifugation, and the fractions were analyzed by SDS-PAGE (upper panel). E1 activity in eluted fractions was assayed (lower panel). One m-unit is equivalent to 1 nmol CO2 released/min. The molecular mass markers used for calibration (in kDa) were: ovalbumin (44), bovine serum albumin (67), human E3 (110), aldolase (158), and catalase (232).
The GroEL-αβ complex with GroEL (26, 48). Moreover, we recently found that the rate of import of the precursor α subunit into mitochondria is markedly faster than its β counterpart. This raises the possibility that part of the α subunit released from the mammalian chaperonin Hsp60 may accumulate in the mitochondrial matrix and exists as assembly competent monomers as suggested by the in vitro data (Fig. 4A). The presence of a free α subunit pool in mitochondria would be advantageous because it ensures a timely discharge of the β subunit from the Hsp60-β complex for assembly with the α, thereby preventing the prolonged “tie-up” of the mitochondrial chaperonin by the β subunit. Further studies are needed to establish this model for chaperonin-mediated biogenesis of hetero-oligomeric protein inside mitochondria.

The GroEL-αβ ternary complex reported here is a novel folding intermediate and differs from the previously described interaction between GroEL and a folding intermediate (Dc) of the Fab fragment (49). The transformation of the disulfide bonded Dc intermediate to the native Fab fragment can occur spontaneously in vitro, but GroEL is capable of binding Dc to form the GroEL-Dc complex (49). Addition of ATP alone leads to discharge of Dc from the GroEL-Dc complex, followed by the conversion of Dc to the functional Fab fragment. In contrast, the GroEL-αβ complex is formed through interaction of the chaperonin with an assembly intermediate comprising two distinct polypeptides. The reconstitution of αβ2 tetramers from the GroEL-αβ complex absolutely requires both GroES and Mg-ATP. Moreover, the binding of the relatively large αβ dimer to GroEL poses a topology problem, because the 85.5-kDa dimer exceeds the 70-kDa size constraint for a protein to reside in the cis cavity of an asymmetric GroEL14-(GroES7)1 complex (9). It is likely that the highly structured αβ dimer and GroES bind to opposite ends of the GroEL double-ring complex to circumvent the size problem associated with the dimer. In line with this trans model, we report in the accompanying paper that a large 86-kDa unfolded maltose-binding protein-α fusion polypeptide on GroEL cannot be sequestered by GroES in a cis configuration (50). Work is in progress to delineate the topology of the GroEL-αβ complex by cryoelectron microscopy.

Fig. 11 depicts our current working model for the chaperonin-mediated biogenesis of hetero-oligomeric protein inside mitochondria.
dissociated from the GroEL-GroES complex, and human mutations on the α subunit that block specific steps in the chaperonin-mediated E1 assembly pathway have been identified (indicated by X).

Dependent E1 assembly pathway. The folding of individual α and β monomers occurs on two separate GroEL scaffolds. The folded assembly competent polypeptides that are released from GroEL readily associate in solution to form the αβ dimeric intermediate, which subsequently dimerizes through obligatorily interactions with GroEL complex to generate the native $\alpha\beta_2$ tetramer. Our earlier pulse-chase labeling studies indicated that the folded and assembled α and β subunits can be observed within 10–20 min after a chase in E. coli (28). This time scale is similar to that observed in vitro, where the assembled α and β subunits first appeared at 45 min of the refolding reaction (Fig. 5). Both dimerization of the αβ intermediate and reconstitution of E1 activity follow the second order reaction with similarly slow rate constants. It is therefore tempting to suggest that the conversion from the dimer to the tetramer is the rate-limiting step in E1 assembly. The αβ dimer dissociated from the GroEL-αβ complex needs to interact again with GroEL/GroES (Fig. 10), similar to that established with refolding of small polypeptides such as rhodanese and citrate synthetase (51, 52), thus perpetuating the cycle until the conversion from dimer to tetramers is complete. Our recent data indicate that the interaction of the αβ dimer with GroEL is complex in that GroEL/GroES and Mg-ATP promote dissociation of the αβ dimer. This results in reassembly of E1 subunits into new αβ dimers with a fraction capable of spontaneously converting to the native $\alpha\beta_2$ tetramer. As described above, the T265R mutation in the α subunit of a classic MSUD patient results in an accumulation of the GroEL-αβ complex by presumably impeding the release of the mutant dimer from GroEL (Fig. 11). Another human mutation, i.e. Y393N in the α subunit that occurs in homozygous affected Mennonite MSUD patients, prevents dimerization of the αβ dimeric intermediate, leading to the production of exclusive mutant E1 dimers both in vitro (data not shown) and in E. coli (28). Characterization of these E1 assembly mutants may shed light on the mechanism for the slow conversion from dimers to tetramers during human E1 assembly.

Acknowledgments—We thank Clay Clark and Carl Frieden for generous gifts of highly purified GroEL preparations, which were used in the initial phase of refolding studies and also served as activity standards for our GroEL preparations. Helpful discussions with Clay Clark concerning GroEL purification are gratefully acknowledged. We also thank Cindy Cote for technical assistance.

REFERENCES

1. Hartl, F. U. (1996) Nature 381, 571–579
2. Bukau, B., and Horwich, A. L. (1998) Cell 92, 351–366
3. Ellis, R. J. (1991) Annu. Rev. Biochem. 60, 321–347
4. Kim, S., Williams, R. K., and Horwich, A. L. (1994) Trends Biochem. Sci. 19, 543–548
5. Viitanen, P. V., Lorimer, G. H., Seetharam, R., Gupta, R. S., Oppenheim, J., Thomas, J. O., and Cowan, N. J. (1992) J. Biol. Chem. 267, 695–698
6. Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1994) Nature 371, 578–586
7. Hunt, J. F., Weaver, A. J., Landry, S. J., Giersch, L., and Deisenhofer, J. (1996) Nature 379, 37–43
8. Mande, S. C., Mehra, V., Bloom, B. R., and Hol, W. G. (1996) Science 271, 161–162
9. Xu, Z., Horwich, A. L., and Sigler, P. B. (1997) Nature 388, 741–750
10. Schmidt, M., Rutkat, K., Rachel, R., Pfeifer, G., Jaenicke, R., Viitanen, P., Lorimer, G., and Buchner, J. (1994) Science 265, 656–659
11. Langer, T., Pfeifer, G., Martin, J., Baumeister, W., and Hartl, F. U. (1992) EMBO J. 11, 4757–4765
12. Weissman, J. S., Hoh, C. M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K., Saibil, H. R., Fenton, W. A., and Horwich, A. L. (1995) Cell 83, 577–587
13. Azem, A., Kessel, M., and Goloubinoff, P. (1994) Science 265, 653–656
14. Todd, M. J., Viitanen, P. V., and Lorimer, G. H. (1994) Science 265, 659–666
15. Llorca, O., Marco, S., Carrascoa, J. L., and Valpuesta, J. M. (1997) FEBS Lett. 405, 195–199
16. Sparrer, H., Rutkat, K., and Buchner, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1196–1100
17. Azem, A., Diamant, S., Kessel, M., Weiss, C., and Goloubinoff, P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12021–12025
18. Zheng, X., Rosenberg, L. E., Kalousek, F., and Fenton, W. A. (1993) J. Biol. Chem. 268, 7489–7493
19. Fisher, M. T. (1995) J. Biol. Chem. 268, 13777–13779
20. Ranson, N. A., Dunster, N. J., Burston, S. G., and Clarke, A. R. (1995) J. Mol. Biol. 250, 581–586
21. Flynn, O. C., Beckers, C. J., Baase, W. A., and Dahlquist, F. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10826–10830
22. Fedorov, A. V., and Baldwin, T. O. (1997) J. Mol. Biol. 268, 712–723
23. Lessard, I. A., and Perham, R. N. (1994) J. Biol. Chem. 269, 10378–10383
24. Reed, L. J., Damuni, Z., and Merryfield, M. L. (1998) Curr. Top. Cell Regul. 27, 41–49
25. Wynn, R. M., Davie, J. R., Zhi, W., Cox, R. P., and Chuang, D. T. (1994) Biochemistry 33, 8962–8968

---

$^{3}$ R. M. Wynn, J.-L. Song, and D. T. Chuang, manuscript in preparation.
GroEL/GroES-dependent αβ Reconstitution

26. Wynn, R. M., Davie, J. R., Cox, R. P., and Chuang, D. T. (1992) J. Biol. Chem. 267, 12400–12403
27. Davie, J. R., Wynn, R. M., Cox, R. P., and Chuang, D. T. (1992) J. Biol. Chem. 267, 16601–16606
28. Wynn, R. M., Davie, J. R., Chuang, J. L., Cote, C. D., and Chuang, D. T. (1998) J. Biol. Chem. 273, 13110–13118
29. Clark, A. C., Hug, E., and Frieden, C. (1996) Biochemistry 35, 5893–5901
30. Bursten, S. G., Ranson, N. A., and Clarke, A. R. (1995) J. Mol. Biol. 249, 138–152
31. Fisher, M. T. (1992) Biochemistry 31, 3955–3963
32. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Vol. 3, pp. A3, Cold Spring Harbor Laboratory, Cold Spring, NY
33. Chuang, D. T. (1988) Methods Enzymol. 166, 146–154
34. Guzman-Verduzco, L. M., and Kupersztch, Y. M. (1987) J. Bacteriol. 169, 5201–5208
35. Hawes, J. W., Schnepf, R. J., Jenkins, A. E., Shimomura, Y., Popov, K. M., and Harris, R. A. (1995) J. Biol. Chem. 270, 31071–31076
36. Mendoza, J. A., Demeler, B., and Horowitz, P. M. (1994) J. Biol. Chem. 269, 2447–2451
37. Mendoza, J. A., Rogers, E., Lorimer, G. H., and Horowitz, P. M. (1991) J. Biol. Chem. 266, 13587–13591
38. Chuang, D. T., and Shih, V. E. (1995) The Metabolic and Molecular Bases of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) 7th Ed., pp. 1239–1277, McGraw-Hill, New York
39. Chuang, J. L., Davie, J. R., Chinsky, J. M., Wynn, R. M., Cox, R. P., and Chuang, D. T. (1995) J. Clin. Invest. 95, 954–963
40. Fisher, C. R., Chuang, J. L., Cox, R. P., Fisher, C. W., Star, R. A., and Chuang, D. T. (1991) J. Clin. Invest. 88, 1034–1037
41. Hu, C. W., Utter, M. F., and Patel, M. S. (1983) J. Biol. Chem. 258, 2315–2320
42. Glerum, D. M., Koerner, T. J., and Tzagolofr, A. (1985) J. Biol. Chem. 270, 15585–15590
43. Glerum, D. M., Muroff, I., Jin, C., and Tzagolofr, A. (1997) J. Biol. Chem. 272, 19088–19094
44. Lister, H., Rudolph, R., and Buchner, J. (1995) J. Mol. Biol. 248, 190–201
45. Ungermann, C., Neupert, W., and Cyn, D. M. (1994) Science 266, 1250–1253
46. Glick, B. S. (1995) Cell 80, 11–14
47. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K., and Hartl, F. U. (1992) Nature 354, 683–689
48. Wynn, R. M., Chuang, J. L., Davie, J. R., Fisher, C. W., Hale, M. A., Cox, R. P., and Chuang, D. T. (1992b) J. Biol. Chem. 267, 1881–1887
49. Langer, T., Lister, H., and Buchner, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8100–8104
50. Huang, Y.-S., and Chuang, D. T. (1999) J. Biol. Chem. 274, 10405–10412
51. Martin, J., Mayhew, M., Langer, T., and Hartl, F. U. (1993) Nature 366, 228–233
52. Weissman, J. S., Kashi, Y., Fenton, W. A., and Horwich, A. L. (1994) Cell 78, 693–702