In Vitro Reconstitution of the 24-meric E2 Inner Core of Bovine Mitochondrial Branched-Chain α-Keto Acid Dehydrogenase Complex: Requirement for Chaperonins GroEL and GroES†

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ABSTRACT: We have investigated the *in vitro* reconstitution of the 24-meric inner core domain (E2c) of the transacylase (E2) component of bovine branched-chain α-keto acid dehydrogenase complex. The yield of recombinant E2c (amino acid residues 161–421 of bovine E2) expressed in *Escherichia coli* was markedly increased by fusing the bacterial maltose-binding protein (MBP) to the amino terminus of bovine E2c. Following factor Xa digestion to remove the MBP moiety, E2c was completely unfolded in 4.5 M guanidine HCl (Gdn·HCl). The denatured E2c monomers (apparent M_r = 27 000) were diluted 100-fold at 25 °C into a refolding buffer containing 5 mM Mg-ATP and a 4-fold molar excess of chaperonins GroEL and GroES. Full E2 activity was recovered in 45 min. Omission of the chaperonins in the refolding buffer failed to recover any E2 activity. Recovery of E2 activity obeyed hyperbolic kinetics as a function of the chaperonin-to-E2c molar ratio and showed a requirement for hydrolysis of Mg-ATP. A stable GroEL–E2c complex was isolated which, in the presence of GroES and Mg-ATP, generated active E2c 24-mers. Dissociation of recombinant E2c 24-mers into active trimers was achieved by incubation in 1.5 M Gdn·HCl at 25 °C. The E2c trimers with an apparent M_r of 84 000 were isolated by sucrose density gradient centrifugation in the presence of the chaotropic reagent. Removal of 1.5 M Gdn·HCl resulted in the spontaneous reassembly of trimers into the native 24-mer structure independent of chaperonins. Our results indicate that *in vitro* refolding of bovine E2c is a chaperonin-mediated process and that spontaneous assembly of the 24-meric structure of E2 proceeds through active trimeric intermediates.

The mammalian branched-chain α-keto acid dehydrogenase (BCKAD)[1] complex catalyzes oxidative decarboxylation of the α-keto acids derived from branched-chain amino acids leucine, isoleucine, and valine. The mitochondrial enzyme complex is organized around a cubic core consisting of 24 transacylase (E2) subunits, to which multiple copies of the decarboxylase (E1), the dehydrogenase (E3), the specific kinase, and the specific phosphatase are attached by ionic interactions (Yeaman, 1989). The E1 component is a heterotetramer ($\alpha_2\beta_2$) with M_r of 46 000 and 35 000 for the E1α and E1β subunits, respectively. The E2 component is a homo 24-mer with monomeric M_r of 46 500, and E3 is a homodimer with monomeric M_r of 55 000. The E2 subunit consists of three folded domains: a lipoyl-bearing, an E1/E3-binding, and an inner core (E2c) domain (N- to C-terminus) connected by flexible hinge regions. The E2 domain structures are highly conserved between α-keto acid dehydrogenase complexes from bacteria to humans (Yeaman, 1989). The three-dimensional structure of the 24-meric inner core domain of the pyruvate dehydrogenase complex from *Azotobacter vinelandii* has been recently determined at 2.6-Å resolution (Mattevi et al., 1992). The structure shows that trimers are the basic functional units of E2 with a 29-Å long active-site channel formed at the interface between two 3-fold related subunits. This topology is very similar to that of the bacterial chloramphenicol acetyltransferase, in which active site histidine residue acts as a general base in the acetyl transfer reaction (Leslie et al., 1988). Eight trimers assemble as a holotruncated cube forming the core of the multienzyme complex.

Our laboratory is interested in the biogenesis and macromolecular assembly of the mammalian BCKAD complex. We have shown previously that overexpression of the bacterial chaperonins GroEL and GroES promotes efficient folding and assembly in *Escherichia coli* of the E1 ($\alpha_2\beta_2$) component of the bovine BCKAD complex (Wynn et al., 1992). In contrast, the core protein, namely, the E2 24-mers of the enzyme complex, can be readily expressed in wild-type *E. coli* without overexpression of chaperonins GroEL and GroES (Griffin et al., 1990). In an earlier study, Willms et al. (1967) showed that the E2 transacetylase 24-mers of the related *E. coli* pyruvate dehydrogenase complex can be dissociated into inactive 70 000-kDa dimers in a dilute solution of acetic acid; renaturation by rapid dilution into a neutral pH buffer containing 2 M urea resulted in restoration of 80–90% of the original catalytic activity. This latter reconstitution, along with our bacterial expression data, raises questions regarding the role of chaperonin proteins in the folding and assembly of E2 multimeric cores of α-keto acid dehydrogenase complexes.

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[1] Abbreviations: E1, branched-chain α-keto acid decarboxylase; E2, dihydrolipoyl transacylase; E3, dihydrolipoyl dehydrogenase; MBP, maltose-binding protein; E2c, E2 inner core domain; Gdn·HCl, guanidine hydrochloride; PEG, poly(ethylene glycol); PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid; AMP-PNP, 5′-adenylyl-β,α-imidodiphosphate; SDS, sodium dodecyl sulfate; hsp, heat-shock protein.

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In the present study, we investigate this problem by overexpressing in *E. coli* the inner core domain (E2c, residues 161–421) of the E2 component of bovine branched-chain α-keto acid dehydrogenase complex. The carboxy-terminal E2c domain contains the active site for the transacylation reaction and the E2 intersubunit binding site, which allows E2 assembly into 24-mers (Chuang et al., 1985). The recombinant E2c protein is completely unfolded in 4.5 M guanidine HCl (Gdn·HCl), as demonstrated by circular dichroism. We show that *in vitro* reconstitution of E2 24-mers is dependent on chaperonins GroEL and GroES. Moreover, we present the direct evidence that enzymatically active E2c trimers exist and may represent the intermediate species for assembly of the properly folded monomers into the 24-meric structure.

MATERIALS AND METHODS

Materials. Reagent grade Tris-HCl, isovaleryl-CoA, lipoic acid, NADH, and malate dehydrogenase were purchased from Sigma. Ultrapure guanidine hydrochloride (Gdn·HCl) was supplied by United States Biochemicals. Crystallographic grade poly(ethylene glycol) (average MW 8000; PEG-8000) was obtained from Hampton Research. Activated, purified serum endoprotease factor Xa was purchased from Boehringer Mannheim. Additional chemicals were of the highest grade commercially available. Rat ATP-citrate lyase was generously supplied by Dr. Paul Srere (Linn et al., 1979), Department of Veteran Affairs Medical Center, Dallas. Chaperonins GroEL and GroES were isolated from lysates of *E. coli* strain CG-712 which carried the multicopy plasmid pGroESL (both generous gifts from Drs. George Lorimer and Anthony Gatenby) (Goloubinoff et al., 1989); GroEL and GroES were purified as previously described (Landry et al., 1991) except that the heat denaturation step at 50 °C was omitted. Amylose resin was prepared from corn starch (Sigma) as described previously (Ferenci et al., 1978), with the exception that cross-linking temperature was maintained at 50 °C, and cross-linking time was decreased to 13 min to increase porosity and improve binding capacity for megadalton-range MBP fusion species. Dihydrolipoamide was prepared from the oxidized form of DL-6,8-thioctic acid amide (Sigma) as described previously (Reed, et al., 1958).

Construction of Expression Vectors. Plasmid pKK-E2c expressing bovine E2c (residues 167–421) was constructed as described previously (Griffin et al., 1990). Plasmid pMALc-E2c, which expressed the MBP–E2c (residues 161–421) fusion protein was constructed as follows: a DNA fragment for the bovine E2c was generated by PCR using an antisense-derived primer containing an engineered *Eco*RI restriction site (underlined) (5′-CTCAGAGAATTCAAGGATGTCTGATCAATC-3′) and a sense-derived primer beginning at amino acid residue 161 (5′-CACGCTGAAATTATGCCACCTCCA-3′). The PCR product was purified with Qiaex resin (Qiagen) and digested with restriction enzyme *Eco*RI. The *Eco*RI/blunt end fragment was ligated into the pMALc plasmid (New England Biolabs) previously digested with *Eco*RI and *Stu*I (blunt end). The nucleotide sequence of the PCR insert was confirmed by DNA sequencing.

Expression and Purification of E2c Protein. E2 inner core domain (E2c) was expressed initially as a fusion protein with maltose-binding protein (MBP) using the New England Biolabs pMALc vector. The plasmid (pMALc-E2c) was transformed into *E. coli* strain XL1-Blue (Stratagene), mutant *E. coli* strain CG-712, or mutant *E. coli* strain CG-712 carrying the compatible expression plasmid pGroESL. Growth media was 2xYT medium (Sambrook et al., 1989) containing 10 mM KCl, 1% glycerol, and antibiotics (100 mg/mL ampicillin, with 17.5 mg/mL tetracycline [XLI-Blue] or 34 mg/mL chloramphenicol [CG-712 + pGroESL]). Cells were grown at 37 or 25 °C (CG-712) for 15–20 h before harvest. Cell pellets were resuspended in buffer A (50 mM potassium phosphate, pH 7.0, 2 mM $MgCl_2$, 0.1% Triton X-100, 1.5 mM NaN_3, 0.1 mM EDTA, 0.1 mM EGTA, 100 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride, and 0.1 mM benzamidine). Cell suspensions were sonicated five times with a Branson sonifier at a power setting of 5 for 30 s using a microprobe. Cell lysates were clarified by centrifugation at 12000*g* for 15 min.

Affinity Purification Column Chromatography. MBP-E2c was purified from *E. coli* lysates with amylose resin affinity columns at low Triton X-100 concentration (0.1%) as described previously (Davie et al., 1992).

Spectrophotometric and Radiochemical E2 Assays. E2 activity was determined spectrophotometrically at 30 °C using a Gilford Response scanning spectrophotometer as modifed from the method described by Angier et al. (1987). The reaction mixture contained 100 mM Tris-HCl, pH 7.5, 20 mM sodium citrate, 10 mM $MgCl_2$, 5 mM ATP, 0.5 mM isovaleryl-CoA, 1 mM dihydrolipoamide, 0.2 mM NADH, 10 units malate dehydrogenase, and 0.1 unit ATP-citrate lyase in a final volume of 0.5 mL. The reaction was initiated by the addition of isovaleryl-CoA and monitored by the decrease in OD at 340 nm. Radiochemical E2 activity assays were performed using [1-^{14}C]isovaleryl-CoA as a substrate as described previously (Chuang, 1988). To assay for the activity of trimers, Gdn·HCl was included in the reaction mixture to a final concentration of 1.5 M to prevent the reassociation into 24-mers.

Sephacryl S-300 HR Gel Filtration. A calibrated Sephacryl S-300HR (Pharmacia) gel filtration column (1.5 cm × 170 cm) was equilibrated in buffer A without EDTA and EGTA. Aliquots (1.8 mL) of either MBP–E2c or free E2c (released by factor Xa digestion) were applied to the S-300HR column. Eluted fractions were collected and analyzed by SDS–polyacrylamide gel electrophoresis and spectrophotometric E2 activity assay, and the fractions containing active, 24-meric E2c species were pooled.

SDS–Polyacrylamide Gel Electrophoresis. Proteins were separated by SDS–polyacrylamide gel electrophoresis essentially as described by Laemmli (1970) using a 10–15% gradient gel with a 4% stacking. Samples were prepared by boiling in the sample buffer for 1 min prior to loading. SDS–polyacrylamide gels were stained with Coomassie brilliant blue.

Unfolding and Refolding Reaction. E2c, MBP–E2c, or full-length E2 (15 μM, monomer) was denatured for 1 h at 25 °C in 150 μL of buffer B (4.5 M Gdn·HCl in 50 mM potassium phosphate, pH 7.0, and 1 mM dithiothreitol). Refolding was initiated at 25 °C by diluting 5 μL of this denatured enzyme solution into 495 μL of refolding buffer C (50 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 600 nM GroEL, 600 nM GroES, and 5 mM ATP). Dilutions were performed by vortexing buffer C (Goldberg et al., 1991) while adding denatured protein solution with a Rainin EDP-Plus motorized pipette. Following dilution, the final Gdn·HCl concentration in the refolding mixture was 45 mM. In positive controls (E2c without Gdn·HCl), Gdn·HCl was added to refolding buffer C to yield a matching final concentration of 45 mM. To stop the refolding reaction at indicated time points, an aliquot (50 μL) was withdrawn, and the reaction was

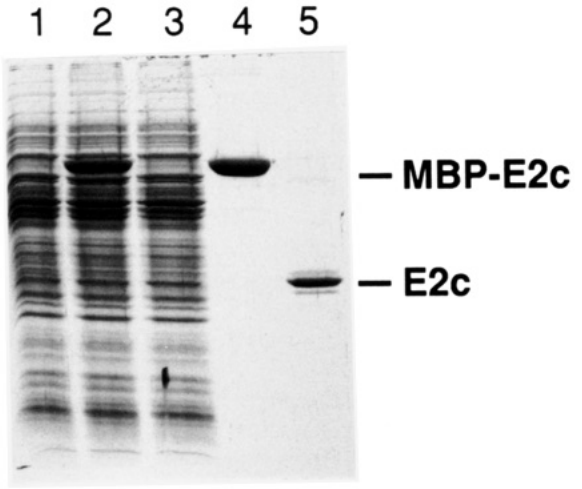

FIGURE 1: Expression and purification of E2c domain. Whole cell lysates from *E. coli* XL1-Blue host strain transformed with either pKK-E2c (E2c residues 167–421, lane 1) or pMALc-E2c (MBP-E2c, residues 161–421, lane 2) are shown for comparison. Amylose affinity purification results in the quantitative removal of MBP-E2c from the lysate (lane 3). Elution of the amylose resin with 10 mM maltose followed by PEG-8000 precipitation (8%) yielded pure MBP-E2c (lane 4). Following factor Xa digestion and S-300HR chromatography, the unconjugated E2c was isolated (lane 5).

terminated by the addition of 50 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA) to chelate Mg^{2+} ions. E2 activity was determined by assaying 50-μL aliquots of the stopped refolding mixture in 0.5 mL of E2 assay solution. For purposes of assigning stoichiometric ratios, the molecular weights used in calculating protein molarities were based on the E2c monomers of 27 kDa (Griffin et al., 1988), GroEL 14-mers of 840 kDa (Hendrix, 1979), and GroES 7-mers of 70 kDa (Chandrasekhar et al., 1986).

Sucrose Density Gradient Centrifugation. Protein complexes were layered upon a 5-mL linear 10–30% sucrose gradient prepared in 50 mM Tris-HCl, pH 7.5, and 0.1 mM dithiothreitol. After centrifugation at 90000*g* for 15–18 h at 4 °C (Beckman SW-50.1 rotor), the gradients were fractionated (330 μL per fraction) and analyzed by SDS–polyacrylamide gel electrophoresis and E2 enzymatic assays. Bovine serum albumin (67 kDa), phosphorylase a (92 kDa), recombinant branched-chain E1 decarboxylase (160 kDa), E2c (648 kDa), and GroEL (840 kDa) were run separately for use as molecular size markers for the gradient.

RESULTS

Expression and Purification of the E2 Inner Core. To study the *in vitro* folding and assembly of E2 24-mers, bovine E2 residues 161–421 (corresponding to the carboxy-terminal E2 inner core [E2c] domain and a portion of the inner core hinge region) were heterologously expressed in *E. coli* using the maltose-binding protein (MBP) fusion expression system. The MBP–E2 inner core fusion protein (MBP–E2c) is expressed at a markedly higher level (Figure 1, lane 2) than the nonfused E2 inner core (E2c) (lane 1), as judged by Coomassie blue staining of the cell lysate. The MBP–E2c protein (Figure 1, lane 4) was isolated with 98% purity from the cell lysate (lane 3) in a single purification step by affinity column chromatography on cross-linked amylose resin. A recovery from the lysate of greater than 95% was achieved. The purified MBP-E2c was subjected to factor Xa digestion, and the cleaved E2c (Figure 1, lane 5) was separated from MBP by Sephacryl S-300HR column chromatography. Retention of enzymatic activity was greater than 95% during

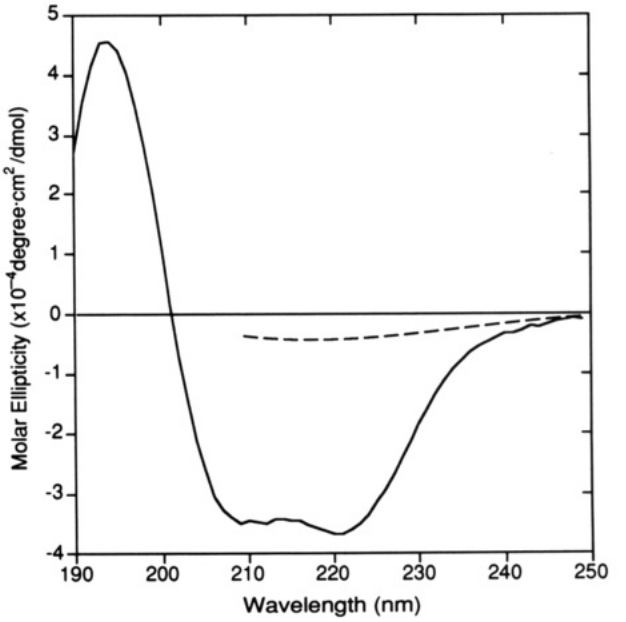

FIGURE 2: Ultraviolet circular dichroism spectra of native and denatured E2c. The CD spectrum of 25 μg/mL of E2c at 25 °C is shown under native conditions (solid line, 50 mM potassium phosphate and 1 mM dithiothreitol) and following denaturation in the same buffer with the addition of 4.5 M Gdn·HCl (dashed line). Spectra represent the average of three scans. Bandwidth was 1.5 nm.

factor Xa digestion and subsequent S-300HR chromatography. The yield of E2c using the MBP–fusion expression system is 50 mg/L of media.

Both MBP–E2c and E2c showed cubic morphology as determined by electron microscopy (data not shown), with dimensions indistinguishable from those of native E2 protein. The results establish that this recombinant protein assembles into 24-mers with octahedral 432 point group symmetry (Chuang et al., 1985). Both MBP–E2c (1.94 units/mg of protein) and E2c (3.76 units/mg of protein) possess transacylase activity as assayed by the ATP citrate lyase-linked spectrophotometric method.

Chaperonins GroEL and GroES-Mediated Refolding of Chemically Denatured E2c. To study the folding and assembly of the E2 domains, the recombinant E2c was denatured in 4.5 M Gdn·HCl (pH 7.5) at 25 °C for 60 min. Under these conditions, the E2c unfolded into a completely "random coil" structure within 10 min as determined by the ultraviolet circular dichroism spectrum (Figure 2). The native, undenatured E2c used as a control reference contained substantial α-helical content.

The Gdn·HCl-denatured E2c had no transacylase activity and migrated as a monomeric species in sucrose density gradient centrifugation. The M_r of E2c monomers is 27 000, as determined by SDS–polyacrylamide gel electrophoresis, or 28 830 as calculated from the amino acid composition. The Gdn·HCl-denatured E2c (15 μM, monomer), when diluted 100-fold into a refolding buffer lacking both chaperonins and Mg-ATP, did not result in recovery of detectable enzymatic activity after 3 h at 25 °C (Figure 3). Additional attempts to refold E2c by dialysis against the refolding buffer did not yield assembled active E2c, even when attempted at 4 °C (data not shown). When denatured E2c (15 μM) was diluted 100-fold into the refolding buffer containing 4-fold molar excess of the chaperonins GroEL/GroES multimers minus ATP, there was a slight (10%) recovery of the transacylase activity during the measured period (15 min to 3 h) (Figure 3). The small recovery of activity is attributed to the presence

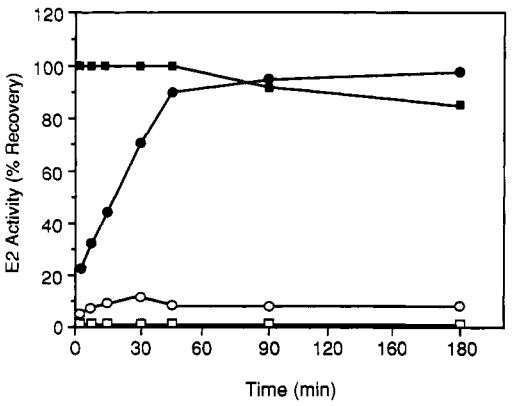

FIGURE 3: Time course of chaperonin dependent refolding of denatured E2c into active enzyme. E2c was unfolded then refolded as described under Materials and Methods. Refolding was studied in the absence or presence of chaperonins GroEL (600 nM, oligomer) and GroES (600 nM, oligomer) and Mg-ATP (5 mM) at 25 °C. The final concentration of monomeric E2c in the refolding reaction mixture was 150 nM in a volume of 0.5 mL. At the indicated times an aliquot was withdrawn and assayed for E2 activity. E2 activity is expressed as a percentage of the activity of an equal amount of undenatured E2, which was 186 milliunits/mL or 100%. (■) Native E2c incubated at 25 °C; (●) denatured E2c + GroEL, GroES, and Mg-ATP; (○) denatured E2c + GroEL, GroES without Mg-ATP; (□) denatured E2c with no addition.

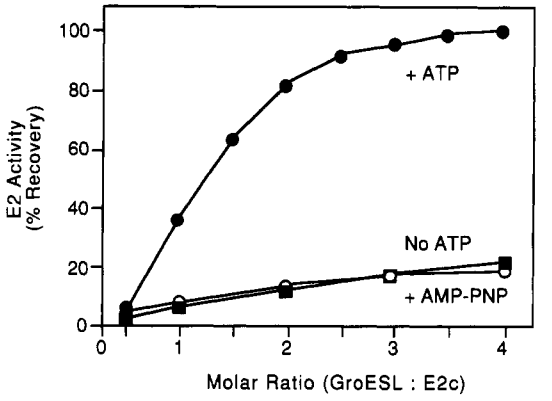

FIGURE 4: Effect of chaperonin concentration on refolding of denatured E2c. Conditions for this experiment were identical to those described in the legend to Figure 3 except that the chaperonin concentration is varied relative to the final monomeric concentration of E2c (150 nM). The mixture contained equimolar GroEL 14-mer, equimolar GroES 7-mer (ranging from 150 to 600 nM oligomeric concentrations), 0.50 M Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 5 mM KCl, and 10 mM ATP. For the experiment with the ATP analog, AMP-PNP, 10 mM concentrations were used. After 1 h of refolding at 25 °C, the samples were assayed for E2 activity. E2 activity is expressed as a percentage of the activity of an equal amount of undenatured E2, which was 174 milliunits/mL or 100%.

of Mg-ATP in the ATP citrate lyase-linked assay for E2 activity. When 5 mM Mg-ATP was included with the chaperonins in the refolding buffer (complete refolding buffer), full (100%) transacylase activity was recovered in approximately 45 min at 25 °C (Figure 3). Native, nondenatured E2c diluted similarly in the refolding buffer served as a positive control and showed a slight decline in activity over 3 h. In parallel experiments using identical conditions, refolding of either denatured MBP–E2c fusion protein or full-length E2 yielded approximately 50% recovery of transacylase activity (data not shown).

The dependence of *in vitro* refolding of denatured E2c upon chaperonins was further investigated by variance of molar ratios of chaperonin protein multimers to E2c monomers. Figure 4 shows that the recovery of E2c transacylase activity obeys hyperbolic kinetics as one increases the ($GroEL_{14}$ + GroES, complex):(E2c monomer) molar ratio. The maximal activity (100% recovery) was attained at a molar ratio of chaperonins to E2c of 4:1 in the complete refolding buffer. The replacement of Mg-ATP with a nonhydrolyzable analog (AMP-PNP) resulted in only 12.5% recovery of enzyme activity compared to that observed with 5 mM Mg-ATP. The low recovery of activity with AMP-PNP is again attributed to the presence of Mg-ATP in the E2 assay mixture. These results suggest that hydrolysis of ATP is essential for chaperonin-mediated folding and assembly.

Isolation of the E2c-GroEL Complex and Kinetics of Conversion to Active Assembled E2c. To isolate the partial folding intermediates, denatured E2c was diluted into the complete refolding buffer at 25 °C. The folding reaction was quenched at different times by addition of 50 mM CDTA, which chelates Mg^{2+} ions. The terminated refolding mixture was subjected to sucrose density gradient centrifugation, and fractions were analyzed by SDS–polyacrylamide electrophoresis and enzyme assay. At 30- and 90-s time points, denatured E2c cosedimented with GroEL (Figure 5A,B) and had no or little demonstrable transacylase activity. The results indicate that denatured E2c forms a stable complex with GroEL which can be isolated. After 5 min of refolding, the E2c was partially released from the GroEL complex, and the folded and assembled E2c 24-mers were catalytically active (Figure 5C). After 20 min of refolding, a greater portion of E2c was dissociated from the complex and assembled into active 24-mers, based on the cosedimentation profile with nondenatured recombinant E2c (Figure 5D). During the time course of 30 s to 20 min, no monomers or putative trimers were observed.

Dissociation of E2c 24-mers into Active Stable Trimers. The inability to trap active trimers in the refolding reaction prompted us to attempt isolation of active trimers by the partial dissociation of E2c 24-mers. Figure 6A shows the sedimentation profile and transacylase activity of the recombinant E2c 24-mer (648 000 Da) after sucrose density gradient centrifugation. The recombinant E2c protein was subjected to dissociation with increasing concentrations of Gdn·HCl for 1 h at 25 °C, and the reaction mixture was analyzed by sucrose density gradient centrifugation and enzyme assay. When exposed to 1.5 M Gdn·HCl concentration, the majority of E2c 24-mers dissociated into a trimeric species of about 84 000 Da in size, as determined by sucrose density gradient centrifugation (Figure 6B). These trimers exhibited transacylase activity when assayed in the presence of 1.5 M Gdn·HCl by the radiochemical method using [1-^{14}C]isovaleryl-CoA and dihydrolipoamide as substrates (Figure 6B). The secondary structure of the trimer in the presence of 1.5 M Gdn·HCl was indistinguishable from the native E2c 24-mers as judged by circular dichroism (data not shown). Spontaneous reassembly of active trimers into 24-mers occurred when 1.5 M Gdn·HCl was partially removed by ultrafiltration, followed by sucrose gradient ultracentrifugation in the absence of the chaotropic reagent (Figure 6C). When the Gdn·HCl was increased to 2.5 M, E2c 24-mers completely dissociated into inactive monomers (data not shown).

DISCUSSION

The present studies on the *in vitro* reconstitution of E2c were facilitated by the overexpression of the MBP–E2c fusion protein in *E. coli*. The presence of the bacterial MBP sequence markedly increased the yield of the fusion protein compared to nonfused E2c in bacteria. Moreover, the MBP sequence provided a convenient affinity ligand for efficient purification

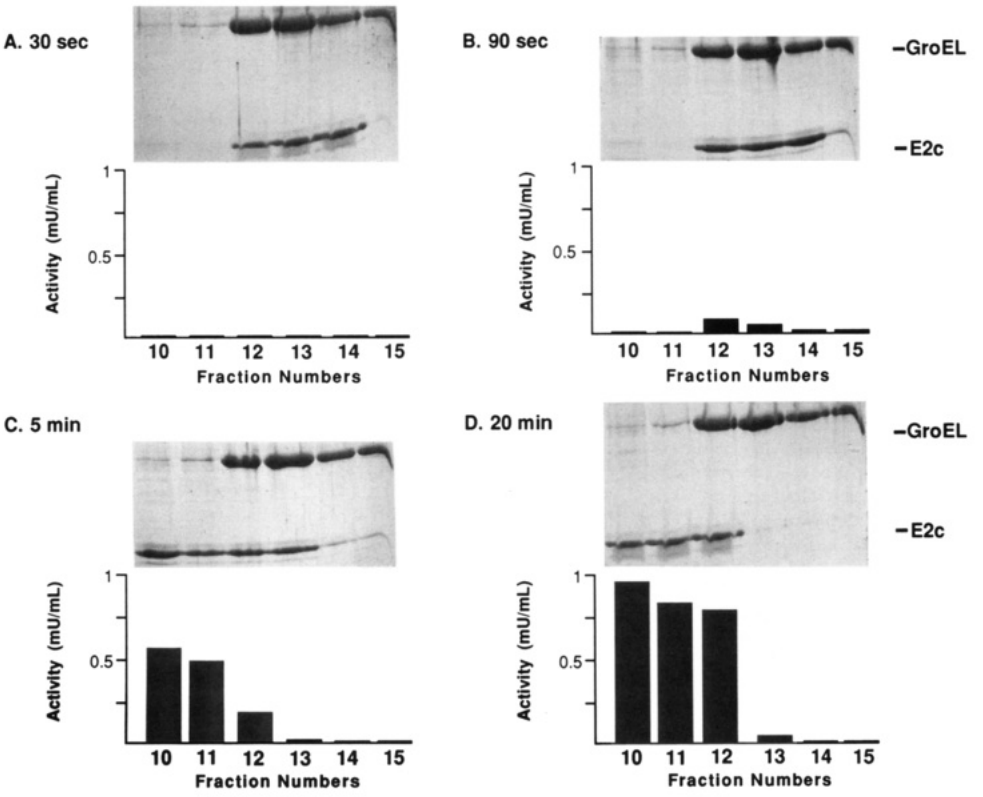

FIGURE 5: Sucrose density gradient isolation of E2c–GroEL complex and conversion to an active assembled E2c 24-mer. Experimental conditions were similar to those described in the legend to Figure 3; however, the reaction was terminated at the indicated times (A, 30 s; B, 90 s; C, 5 min; D, 20 min) by the addition of 50 mM CDTA to chelate Mg^{2+} ions. The 0.5-mL reaction mixture was then subjected to a 10–30% sucrose density gradient centrifugation (volume: 5 mL) using an SW-50.1 Beckman rotor. Proteins were separated at 90000*g* at 4 °C for 15 h. The gradient was fractionated into 15 fractions (330 μL each), which were assayed for enzyme activity and analyzed by SDS–polyacrylamide gel electrophoresis and Coomassie blue staining.

by amylose resin affinity chromatography and appeared to have no effect on E2c 24-meric assembly, structure, or function. Removal of MBP moiety was achieved by digestion with a specific endoprotease factor Xa with minimal loss of enzymatic activity.

To carry out *in vitro* refolding and assembly, the E2c was completely unfolded in 4.5 M Gdn·HCl as determined by circular dichroism spectra. Upon dilution of the denaturant, the E2c failed to refold into active species at 25 °C in the absence of chaperonin proteins. Lowering the temperature to 4 °C for 15–18 h did not result in spontaneous refolding (data not shown). This is in contrast to previously reported observations that significant spontaneous refolding occurs at lowered temperatures with denatured rhodanese [80% refolding at 10°C (Mendoza et al., 1991)] and ribulose 1,5-bisphosphate carboxylase [50% refolding at 15 °C (Viitanen et al., 1990)]. The addition of Mg-ATP and the chaperonin GroEL and GroES to the refolding buffer allowed full reconstitution of enzyme activity for E2c. The assembly of refolded E2c into 24-meric structure is indicated by recovery of enzymatic activity and the sedimentation profiles on sucrose density gradient centrifugation. Thus, our results clearly establish that *in vitro* reconstitution of the E2c domain of the branched-chain α-keto acid dehydrogenase complex is a chaperonin-mediated process that requires GroES-regulated hydrolysis of Mg-ATP (Ellis et al., 1991; Gething et al., 1992; Hendrick et al., 1993; Wynn et al., 1994). As described above, it has been shown that the acid-dissociated, inactive E2 dimer of the *E. coli* pyruvate dehydrogenase complex can be reconstituted into an active 24-mer with high yield in the absence of chaperonin proteins (Willms et al., 1967). However, the presence of a dimeric species following thedissociation reaction with dilute acetic acid indicates that the E2 transacetylase may not have been completely unfolded under these conditions. The results of this earlier study may be related to partial dissociation and reassociation of the E2 transacetylase.

The present study shows that the carboxy-terminal domain of E2 can be properly folded *in vitro* independent of other amino-terminal domains with recovery of 100% activity. Whether these results apply *in vivo* to the entire polypeptide remains to be determined. Our preliminary data indicate that refolding *in vitro* of the full-length E2 produces only 50% recovery of the original E2 activity. This result suggests that upstream domains (i.e., lipoyl bearing and E1/E3 binding) may affect folding efficiency of the downstream E2c domain. The homo 24-meric structure of the reconstituted E2c domain represents the largest number of subunits that have been refolded and assembled *in vitro* to date with the aid of chaperonin proteins. The E2c polypeptide is enzymatically inactive when complexed with GroEL (Figure 5A). The folding of the E2c polypeptide is facilitated by the scaffold provided by GroEL. The reaction cycle of GroEL and GroES has been recently described (Martin et al., 1993). Binding of the unfolded polypeptide within the cavity of GroEL promotes ADP and GroES release. Upon ADP–ATP exchange, GroES reassociates with GroEL. Subsequent ATP hydrolysis releases the bound polypeptide for folding. A partially folded polypeptide rebinds to GroEL and the cycle repeats until the folding process is complete.

The assembled, active E2c 24-mer is not associated with GroEL as shown in Figure 5D. A question arises as to whether oligomeric assembly takes place before or after release of the folded monomers from GroEL. The current dogma suggests that each GroEL 14-mer can accommodate only one or two polypeptide substrates of 35–40 kDa in the central cavity

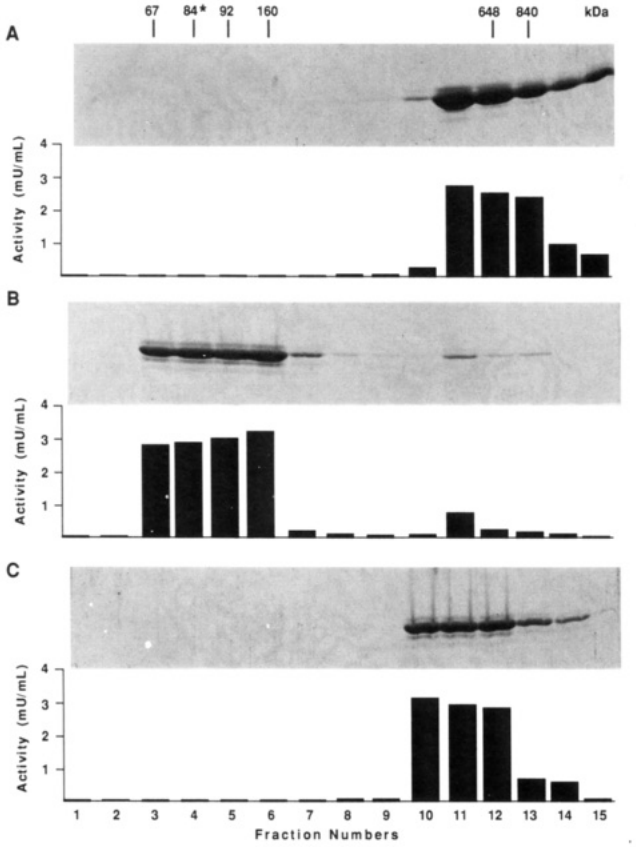

FIGURE 6: Dissociation of E2c 24-mers into active trimers and reassembly into 24-mers. Panel A shows the native (nondenatured) E2c migration on a 10–30% sucrose density gradient. Enzyme concentration was 250 μg/mL initially. Aliquots (10–15 μL) of each fraction (330 μL) were used for the enzyme assays and SDS–polyacrylamide gel electrophoresis. Panel B shows the result of the addition of 1.5 M Gdn·HCl to the native enzyme followed by separation on a 10%–30% sucrose gradient containing 1.5 M Gdn·HCl. Enzyme concentrations were the same as above. Panel C indicates the reassembly of the trimers following partial removal of Gdn·HCl and sucrose by Centriprep-30 (Amicon) concentration. The enzyme was separated on a 10–30% sucrose gradient without Gdn·HCl. Radiochemical assays were performed using [1-^{14}C]isovaleryl-CoA as the substrate. Molecular size markers are indicated for bovine serum albumin (67 kDa), phosphorylase a (92 kDa), E1 decarboxylase (160 kDa), E2c (648 kDa), and GroEL (840 kDa). The M_r of E2c trimers (84 kDa) derived from a standard curve is shown by an asterisk.

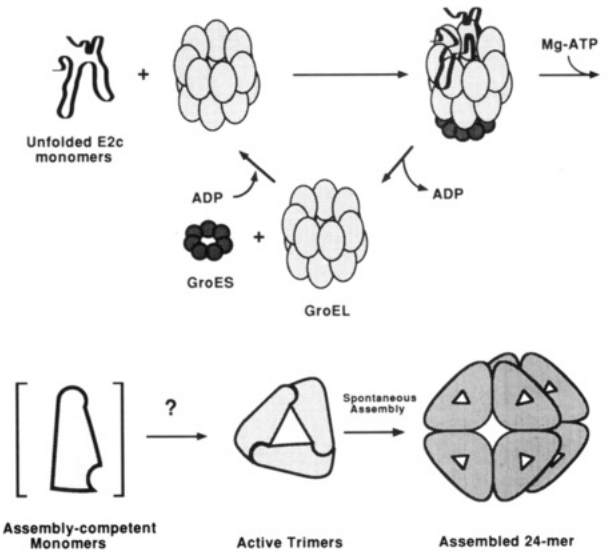

FIGURE 7: Proposed pathway for in vitro reconstitution of the 24-meric structure of the E2c domain. The refolding of the denatured E2c monomers begins with binding to the open end of the GroEL 14-mer complexed with the GroES 7-mer. Refolding occurs within the central cavity of the GroEL cylinder (Martin et al., 1993) Multiple rounds of Mg-ATP hydrolysis result in the release of assembly-competent monomers (shown in a bracket). Chaperonins GroEL and GroES recycle in the presence of ADP to form a second chaperonin–polypeptide complex. The monomers assemble into an intermediate species of active trimers whereby constitutive polypeptides assume their final conformation. Active trimers interlock through carboxy-terminal hydrophobic knobs (Mattevi et al., 1992) to produce the native 24-mer structure with octahedral symmetry. The mechanism for the assembly of monomers into active trimers is not known, as depicted by a question mark. However, the present *in vitro* reassociation experiment establishes that the assembly of the trimeric intermediates into active 24-mers is a spontaneous process independent of chaperonins.

(Langer et al., 1992; Bochkareva et al., 1992). Thus, spatial constraints would not accommodate an oligomeric assembly when the folded peptide is still attached to GroEL. Moreover, it is difficult to picture how multiple nascent E2c chains would be recognized by a single GroEL scaffold for oligomeric assembly to occur *in situ*. Recent studies with mitochondrial ornithine transcarbamylase suggest a mechanism in which chaperonin GroEL and GroES facilitate folding of assembly-competent but inactive monomers; the properly folded monomers then undergo spontaneous assembly to form active trimers (Zheng et al., 1993).

We next asked the question: Is there an intermediate oligomeric species during the assembly of the monomer into the final E2c 24-meric structure? It has been speculated that the E2 core of α-keto acid dehydrogenase complexes is assembled from trimers. This was supported by earlier electron microscopy data, which showed that the E2 component of *E. coli* pyruvate dehydrogenase complex (Willms et al., 1967) and bovine branched-chain α-keto acid dehydrogenase complex (Chuang et al., 1985) possess octahedral symmetry with eight trimers occupying the vertices of a truncated cube. This structure is substantiated by the results of a recent crystallographic study of the E2c (transacetylase) of the pyruvate dehydrogenase complex from *A. vinelandii* (Mattevi et al., 1992). Within this structure, the most extensive interactions (involving about 25% of the monomer-accessible surface) occur within individual trimers. Upon oligomerization of the trimers to form a truncated cube, only an additional 8% of the monomer-accessible surface is buried. We attempted to isolate the putative trimeric species of E2c during chaperonin-mediated refolding by quenching the reaction at different times with CDTA. However, only active 24-mers, but no trimers, were detected by sucrose density ultracentrifugation (Figure 5). The difficulty may be that the CDTA-quenched refolding mixture was subjected to an 18-h centrifugation, prior to analysis by SDS–polyacrylamide gel electrophoresis and enzyme assay. During this time, the putative trimeric intermediates may have assembled into 24-mers (Figure 6C).

We therefore undertook an indirect approach by carrying out a partial dissociation of recombinant E2c. This experiment utilized the mild chaotropic conditions of 1.5 M Gdn·HCl,-which promoted disruption of the relatively weak interactions between neighboring trimers. The stronger interactions between the two 3-fold related subunits required for E2 activity were not perturbed. Results of the radiochemical assay demonstrated unequivocally the existence of an active trimeric species of E2c (apparent M_r = 84 000), confirming the basic functional unit deduced from X-ray crystallographic structure (Mattevi et al., 1992). The inclusion of 1.5 M Gdn·HCl in the reaction mixture ensured that reassociation of trimers

into active 24-mers did not occur during the enzyme assay. Other active partially dissociated E2 species have been previously described. Treatment of the E2 transacetylase of the *E. coli* pyruvate dehydrogenase complex with *N*-acetylimidazole was previously shown to produce an active smaller species of E2, probably a trimer. The latter was capable of binding E1 and E3 to produce an active pyruvate dehydrogenase complex with an apparent M_r of about 500 000 (Schwartz & Reed, 1969). The E2 transacetylase of the pyruvate dehydrogenase complex from *A. vinelandii* was shown to dissociate into a smaller tetrameric species in the presence of Gdn·HCl (Hanemaaijer et al., 1989). In the present study, removal of 1.5 M Gdn·HCl resulted in a spontaneous reassembly of active trimers into 24-mers as determined by sucrose density ultracentrifugation (Figure 6C). Our results, therefore, support the model depicted in Figure 7, in which chaperonins GroEL and GroES mediate the folding of assembly-competent monomers. The monomers assemble into an intermediate active trimeric species, where the role of chaperonins is undetermined, as assembly-component monomers of E2c thus far cannot be isolated for oligomerization studies. The assembly of trimers into 24-mers is apparently spontaneous and independent of chaperones.

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