Intramitochondrial Folding and Assembly of Medium-chain Acyl-CoA Dehydrogenase (MCAD)

DEMONSTRATION OF IMPAIRED TRANSFER OF K304E-VARIANT MCAD FROM ITS COMPLEX WITH hsp60 TO THE NATIVE TETRAMER*

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We incubated in vitro translated precursor of medium-chain acyl-CoA dehydrogenase (MCAD) with isolated rat liver mitochondria and fractionated the solubilized mitochondria on gel filtration. After a 5-min import into mitochondria, MCAD was recovered exclusively as a high molecular weight (hm3) complex (700,000), while after a 10-min import, it was recovered mainly in the hm2 complex and mature tetramer, with a small amount in monomer. Either a further 15-min chase or exposure to ATP caused a marked decrease of MCAD in the hm2 complex and an increase in the mature tetramer in comparable amounts, suggesting that the hm2 complex was the precursor of tetramer. No monomer was detected in either case. Using specific antibodies, we have shown that the hm1 complex represented a complex of MCAD and heat-shock protein 60 (hsp60), and, that upon import into mitochondria, unfolded MCAD first formed a transient complex with mitochondrial heat-shock protein 70 (hsp70mt) and then transferred to hsp60 to complete its folding into an assembly-competent conformation. We also examined the assembly of K304E MCAD, which is a prevalent variant enzyme among patients with MCAD deficiency. The assembly of the K304E into its tetrameric form was severely impaired. The binding of K304E with hsp70mt and its transfer from hsp70mt to hsp60 were normal. However, the hsp60 complex of K304E was much more stable than the wild-type counterpart upon a 15-min chase or exposure to ATP, suggesting that the folding in, or the transfer of K304E subunit to tetramer from, the complex with hsp60 was impaired.

Medium-chain acyl-CoA dehydrogenase (MCAD)1 is a mitochondrial enzyme catalyzing the first step of b-oxidation of fatty acids with medium chain length (1). Human MCAD is synthesized in the cytosol as a 421-amino acid (46.6 kDa) precursor protein with a 25-amino acid leader peptide at the N terminus (2-4). The precursor (p) MCAD is imported into mitochondria and translocated into the matrix, where its leader peptide is proteolytically cleaved, producing the 396-amino acid (43.6 kDa) mature protein. The monomeric enzyme is then assembled into the native and biologically active homotetrameric form (2). Recent x-ray crystallographic studies indicate that MCAD-tetramer is actually a dimer of two dimers. Domains involved in the monomer-monomer interaction are different from those involved in the dimer-dimer interaction (5).

Hereditary MCAD deficiency is mainly detected among Caucasian children of northwestern European origin (6). It causes episodic vomiting and hypoketotic hypoglycemia, and, if not treated, the patients may die. The incidence of MCAD deficiency is relatively high for a genetic metabolic disorder. Among MCAD-deficient patients, an A to G transition at position 985 in the coding region of the gene is a highly prevalent mutation, which is found in 89% of all variant MCAD alleles. This mutation results in a glutamate being substituted for the normal lysine at position 304 (K304E) of the mature MCAD. This K304E-variant of MCAD is unstable and it is undetectable in patients’ tissues (7). Since lysine 304 is located in the critical domain involved in the normal dimer-dimer interaction in the tetramer assembly (5, 8), it has been suggested that this change of the charge at the interface of subunits causes an impairment of the normal assembly of variant subunits into a tetramer, thereby causing instability of the protein (9-11).

We have recently studied the tetrameric assembly process of normal human MCAD and three of its variants, each of the latter containing an acidic or basic substitution of lysine 304 including K304E (12). Each of the variants was produced via site-directed mutagenesis and then examined by in vitro expression in the presence of isolated rat liver mitochondria. For each protein, we analyzed the molecular forms of MCAD that were imported into the isolated mitochondria using gel filtration at different times following the import reaction. Three forms of MCAD-related proteins of different size were detected. These included the monomer (44 kDa), the mature tetramer (176 kDa), and, finally, a high molecular weight complex (hm2; ~700,000). No dimer was detectable. The precise nature of this hm2 complex was unknown. Time course studies demonstrated that tetrameric assembly of acidic variants was impaired. Furthermore, our kinetic analysis indicated that the hm2 complex, and not the free monomer, represented the immediate precursor of the mature tetramer, both in the wild-type and variant forms of MCAD.

Recent studies have demonstrated a class of proteins referred to as molecular chaperones, which facilitate various steps of protein synthesis (13, 14). Relevant examples include members of the hsp70 and hsp60 families of stress proteins which may work in tandem to achieve protein folding and/or assembly (15-17). In the case of mitochondria, proteins entering into the organelle appear to first bind to mitochondrial form
of hsp70 (hsp70\textsubscript{mit}). Such an interaction is believed to prevent the premature folding of the incoming polypeptide. Once entirely translocated into the organelle, the newly imported protein is transferred to the large oligomeric hsp60 complex which presumably functions to facilitate the folding of the protein and perhaps orchestrate oligomeric assembly.

Our observation that a portion of newly imported MCAD was found within a hMr, complex, along with the known characteristics of molecular chaperones like hsp70\textsubscript{mit} and hsp60, prompted our interest in the nature and role of the hMr, complex in the assembly process of MCAD. In the present study, the identity of the hMr, complex and its potential role in the assembly of tetrmeric MCAD, be it the wild-type or K304E-variant, were studied.

**EXPERIMENTAL PROCEDURES**

**Materials**—The entire coding region of normal human pMCAD cDNA was amplified using the polymerase chain reaction (PCR) and cloned into pBluescript vector (Stratagene). Variant pMCAD cDNA with G\textsuperscript{396}, encoding Kp329E\textsubscript{2} (K304E), was created by site-directed mutagenesis as previously described (12). Antibodies against the mitochondria-bound hsp60 proteins were prepared and characterized as described previously (15, 19).

**In Vitro Transcription/Translation of pMCAD cDNA**—In vitro transcription of the wild-type and variant pMCAD cDNAs in pBluescript was performed using T7 RNA polymerase (Pharmacia LKB Biotechnolo-gy Inc.) according to the protocol of the manufacturer. In vitro translation was performed using a rabbit reticulocyte lysate translation system (Promega) and \((\text{3S})\)methionine (Amersham) according to the manufacturer's instructions.

**Gel Filtration Analysis of Newly Imported Wild-type and K304E-variant MCADs**—Rat liver mitochondria were prepared from male Wister rats as previously described (20) and suspended in HMS buffer (2 mm Hepes, pH 7.4, 220 mm o-mannitol, and 70 mm sucrose) at 20 mg of protein/ml. Import mixture containing 200 ml of in vitro translation product and 100 ml of rat liver mitochondria was incubated at 30 °C for a period of 5 or 10 min. The mixture was then treated with 2.5 ml of 4 mg/ml trypsin inhibitor for 10 min. The mitochondrial pellet was washed twice with HMS buffer and dissolved in 100 ml of solubilization buffer (10 mm potassium phosphate buffer, pH 8.0, 0.5 mm EDTA, 1% Triton X-100, and 0.4 mg/ml trypsin inhibitor). Solubilized mitochondria were centrifuged at 15,000 × g for 20 min, and the supernatant was recovered.

For a pulse-chase study of the newly imported MCAD, the import mixture was first incubated at 30 °C for 10 min (pulsed). Trypsin-treated mitochondria were washed twice and suspended in 100 ml of HMS buffer. The mitochondrial suspension was further incubated at 30 °C for 15 min (chased). The mitochondrial pellet was solubilized as described and centrifuged.

For gel filtration, a Sepharcl\textregistered 300 HR (Pharmacia) column (0.7 × 16 cm) was equilibrated with running buffer (10 mm potassium phosphate buffer, pH 8.0, and 0.5 mm EDTA) and was calibrated with protein standards thyroglobulin (670 kDa), y-globulin (158 kDa), and ovalbumin (44 kDa). The void volume of the column was determined via the elution of blue dextran. The supernatant of the solubilized mitochondria was applied to the column after centrifugation, and eluate was collected in an equal volume of acetone, and the precipitates were resuspended in 40 ml of sample buffer (0.6 mm Tris, pH 8.85, 2% sodium dodecyl sulfate (SDS), 5% \(\beta\)-mercaptoethanol, 10% glycerol, and bromphenol blue). A 20-ml aliquot was analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (21). Labeled MCAD bands were visualized by autoradiography, and the intensity of the MCAD bands on the x-ray film was determined densitometrically using a Bio-Image system (Millipore).

**Effects of Apyrase and ATP on Tetratmer Assembly**—200 ml of in vitro translation product were incubated with 100 ml of rat liver mitochondria at 30 °C for 10 min. Trypsin-treated mitochondria were solubilized in 100 ml of the solubilization buffer and centrifuged at 15,000 × g for 20 min. The supernatant was diluted with 100 ml of 50 mm potassium phosphate buffer, pH 8.0, 5 mm MgCl\textsubscript{2}, and 2 mm NADH and incubated at 30 °C for 15 min with either 20 units/ml apyrase (Sigma, grade VIII) or 2.5 mm ATP. The sample was applied to the gel filtration column, and the eluate in each fraction was analyzed using SDS-PAGE and fluorography.

**Immunoochemical Analysis of Gel Filtration Eluate for MCAD-hsp60 Complex Using Anti-hsp60 Antibody**—After a 10-min import, solubilized mitochondria were fractionated by gel filtration. Fractions, each corresponding to the peak of hMr, complex or tetramer (fraction 3 or 6, respectively), were adjusted to a final volume of 1.5 ml with TETN buffer (25 mm Tris-HCl, pH 7.5, 5 mm EDTA, 250 mm NaCl, and 0.1% Triton X-100), and each fraction was divided into three aliquots. To each aliquot 0, 5, or 10 ml of anti-hsp60 antisera were added, and the difference in the amount of added antisera in the first two tubes was made up to the total of 10 ml with normal rabbit serum. The samples were incubated at 4 °C for 30 min by rotating end-over-end. After 10 ml of 10% Staph A (Life Technologies Inc.) were then added. After 15 min of incubation at 4 °C, Staph A cells were sedimented by centrifugation and washed twice with TETN buffer, followed by two washes with 10 mm Tris-HCl, pH 7.5, and 5 mm EDTA. Staph A-immunoglobulin-antigen complexes were dissociated by boiling for 3 min in 30 ml of the sample buffer. Proteins in the supernatant after the first centrifugation were acetone-precipitated and redisolved in 30 ml of the sample buffer. For radiolabeled MCAD, 25 ml of the immunoprecipitated proteins or 12.5 ml of the acetonem precipitated proteins from the supernatant were analyzed by SDS-PAGE and fluorography. To quantify hsp60, 1 ml of each sample was applied to SDS-PAGE, and proteins were electroblotted to a nitrocellulose-P membrane (Millipore) according to the method of Tow- bin et al. (22) and probed with anti-hsp60 antisera using a Protoblot Western Blot AP system (Promega). Intensity of MCAD band on the autoradiogram and hsp60 band on the immunoblot was measured using a densitometer, and it was expressed in densitometric units (units\textsubscript{a}), respective MCAD. The quantity of hsp60 bound to hsp60 was calculated from the amount of MCAD (units\textsubscript{a}) bound to per unit of hsp60 in the immunoprecipitated fraction.

**Immunoochemical Analysis of MCAD Imported into Mitochondria and Complexed with hsp60 or hsp70\textsubscript{mit} Using Specific Antibodies**—For the analysis of mitochondrial MCAD complexed with hsp60, 100 ml of in vitro translation product were incubated with 50 ml of rat liver mitochondria at 30 °C for 10 min. The mixture was treated with trypsin followed by the addition of trypsin inhibitor as described above. Isolated mitochondria were suspended in 200 ml of HMS buffer and were divided into two equal aliquots. Mitochondria in one aliquot were immediately pelleted and solubilized in 100 ml of the solubilization buffer, and the other aliquot was further incubated at 30 °C for 15 min before centrifugation and solubilization. Solubilized mitochondria were centrifuged at 15,000 × g for 20 min. The supernatant was divided into two 50-ml aliquots, and the volume was adjusted to 0.5 ml with TETN buffer. To each tube, either 5 ml of normal rabbit serum or 5 ml of anti-hsp60 antiserum were added. Immunoprecipitation and detection of MCAD and hsp60 were performed as detailed above.

In order to study the sequential action of hsp70\textsubscript{mit} and hsp60 in the process of intramitochondrial folding and assembly of MCAD, 50 ml of in vitro translation product were mixed with 25 ml of rat liver mitochondria in 1% Triton X-100, and the mixture was incubated at 30 °C for 25 min. The sample was then divided into three equal aliquots, and 5 ml of anti-hsp60 antisera were added. 5 ml of anti-hsp60 antisera were added to the other tube. Immunoprecipitation and detection of labeled MCAD in the resulting precipitates were performed as described above.

**RESULTS**

**Time Course of Tetratmer Assembly of Wild-type MCAD in Mitochondria**—We first incubated in vitro translated, radiolabeled wild-type pMCAD with isolated mitochondria at 30 °C for 5 min. After removing all the pMCAD attached to the outside surface by treating with trypsin, mitochondria were solubilized by addition of Triton X-100. After centrifugation, the supernatant was analyzed by gel filtration. Radiolabeled wild-type MCAD eluted predominantly as hMr, complex from the gel filtration column. The size of this hMr, complex was approxi-
Effects of ATP and Apyrase on the hM Complex—Previous studies have shown that the mitochondrial hsp60 proteins are intimately involved in the folding and assembly of newly imported proteins within the mitochondria (23, 24). Since the subunit size of hsp60 protein is 60 kDa and hsp60 exists as single or double heptamer-toroidal rings (25), the size of the hM complex was close to that expected of a hsp60 complex containing MCAD. Thus, we investigated whether the observed hM complex of newly imported MCAD might represent a MCAD-hsp60 intermediate. Since the release of proteins from hsp60 is known to be caused by ATP (24), we examined the effects of either ATP depletion (via the enzyme apyrase) or ATP addition on MCAD assembly in mitochondria. After a 10-min import of MCAD followed by solubilization, mitochondria were further incubated for 15 min at 30 °C with either apyrase or ATP, and the reaction mixture was analyzed by gel filtration. In the mitochondrial lysate incubated with apyrase, 55% and 39% of MCAD eluted as hM complex and tetramer, respectively (Fig. 2, shown in solid circles with solid line). In contrast, when the mitochondrial lysate was incubated with ATP, 21% and 75% of MCAD eluted as the hM complex or tetramer, respectively (Fig. 2, shown in open circles with dotted line). These results indicated that the transfer of MCAD from the hM complex to tetramer was ATP-dependent. Note that essentially no monomer was observed after exposure of the mitochondrial lysate to ATP.

Identification of the hM Complex as a Complex of MCAD and hsp60—In order to test whether the hM complex represented a complex of MCAD and hsp60, the fraction containing hM complex (tube 3: ~700,000) was examined by immunoprecipitation with an antibody specific to hsp60 (Fig. 3). We also tested the tetramer fraction (tube 6: 160,000) with the same antibody as a negative control. In the hM complex fraction, MCAD co-precipitated with hsp60. As the amount of anti-hsp60 antibody increased, the amount of residual MCAD in the supernatant decreased, while that of MCAD in the precipitates increased. With the highest amount (10 μl) of anti-hsp60 antibody used, the amount of MCAD in the supernatant decreased by 57%, while 40% was recovered as isolated precipitate. When the tetramer fraction (tube 6) was treated with the same anti-hsp60 antibody in a similar manner, essentially all of MCAD remained in the supernatant, while no MCAD precipitated at all regardless of the amount of the antibody used.

We also determined the relative amount of hsp60 in tube 3 (~700,000; containing the hM complex) and tube 6 (160,000; containing the tetramer) using immunoblot and densitometry. The ratio of the amount of hsp60 in tube 3 and that in tube 6 was 1.0 to 0.59, indicating that a considerable portion of hsp60 in mitochondria was in forms other than single or double heptamer-toroidal rings. When the amount of hsp60 was quantified using immunoblot analysis with and without treatment...
with 10 μl of anti-hsp60 antibody, 97% and 92% of hsp60 protein were precipitated in tubes 3 and 6, respectively (data not shown), indicating that with the use of 10 μl of the antibody, hsp60 was almost quantitatively precipitated. Thus, these data suggested that the MCAD in the hMr complex fraction (43% of the total), that was not precipitated with anti-hsp60 antibody, represented macromolecular complex of MCAD other than that with hsp60, such as aggregates of itself or complexes with other proteins.

Interaction of K304E-variant MCAD and hsp60 in Mitochondria—In order to examine the maturation of the K304E-variant MCAD, a pulse-chase experiment was performed exactly as described in Fig. 1 for the wild-type MCAD. Following a 5-min import reaction, the vast majority (72%) of the K304E MCAD protein appeared to be severely impaired. It was also notable that a significant amount of K304E monomer was detectable not only after a 10-min import alone, but also with a subsequent 15-min chase.

Next, we tested the effects of apyrase and ATP on the K304E-variant MCAD-hsp60 complex as was done earlier to the wild-type MCAD-hsp60 complex. In the mitochondrial lysate incubated for 15 min with apyrase, the variant MCAD eluted exclusively within the hMr complex (Fig. 5, shown with solid circles and solid line). When the lysate was incubated with ATP, 64% of K304E still remained as hMr complex. The remainder of the K304E eluted mostly as a tetramer, with only a small amount detected within the monomer fraction (Fig. 5, shown with open circles and dotted line).

Our previous studies indicated that both the rate and overall amount of transfer of the K304E-variant MCAD from the hMr complex into its final and mature tetrameric form were markedly less than those observed for the wild-type protein. This observation, together with the consideration for the molecular size of the complex, provided the first clue that the hMr complex represents the complex of MCAD and hsp60 (12). In this study, we accurately compared the amount of the wild-type MCAD associated with hsp60 in the hMr complex and that of K304E-variant before and after the chase reaction using anti-hsp60 antibody. After the in vitro translated pMCAD protein was incubated with mitochondria, the mitochondria were solubilized and treated with anti-hsp60 antibody. After a 10-min incubation, both the wild-type and variant MCAD proteins in the mitochondria co-precipitated with hsp60 in similar amounts, 3.9 and 4.5 units per unit of hsp60, respectively (Fig. 6). After a 10-min import and subsequent 15-min chase period, only 0.2 units of the wild-type MCAD co-precipitated with a unit of hsp60, indicating that the majority of the wild-type was released (transferred) from the complex during the 15-min chase.

Fig. 4. Gel filtration of newly imported K304E MCAD in rat liver mitochondria. In vitro translation product containing K304E pMCAD was incubated with rat liver mitochondria at 30 °C for 5 or 10 min. After trypsin treatment, mitochondria were immediately solubilized or further incubated for 15 min at 30 °C before solubilization. The following procedures were performed exactly as detailed in the legend to Fig. 1. Symbols are: ○, 5-min import; ●, 10-min import; ●—●, 15-min chase after 10-min import.
period. In contrast, even after the 15-min chase period, 2.0 unit$_{R}$ of the variant MCAD still co-precipitated per unit$_{I}$ of hsp60, indicating that the rate of release (transfer) of the variant from the complex was severely retarded.

Role of Mitochondrial hsp70 (hsp70$_{mit}$) in the Intramitochondrial Folding and Assembly of MCAD—Recent studies have shown that some proteins, which are newly imported into mitochondria, first interact with hsp70$_{mit}$. The interaction appears to occur in the course of translocation into mitochondria (26, 27). The loosely folded mature subunits are released from hsp70$_{mit}$ in an ATP-dependent manner, and the released subunits form a complex with hsp60 prior to complete folding and oligomer assembly (15, 16, 28). Therefore, we studied the interaction of the wild-type and K304E-variant MCADs with hsp70$_{mit}$ and their transfer from hsp70$_{mit}$ to hsp60. After varying periods of import, mitochondria were treated with trypsin removing pMCAD attached to the outside surface. Mitochondria were then solubilized in a buffer containing apyrase and divided into two equal aliquots. Each aliquot was immunoprecipitated with either anti-hsp70$_{mit}$ antibody or anti-hsp60 antibody (Fig. 7). After a 10-min incubation at 30 °C, both the wild-type and the variant co-precipitated with hsp60, but not with hsp70$_{mit}$ (data not shown). After a 5-min or 10-min incubation at 16 °C, however, a large amount of wild-type MCAD was complexed with hsp70$_{mit}$, presumably because at the low temperature the translocation of the precursor MCAD is arrested at the mitochondrial contact site. When the temperature was raised to 30 °C, wild-type MCAD was completely transferred from hsp70$_{mit}$ to hsp60 within 10 min. The interaction of K304E-variant MCAD with hsp70$_{mit}$ was similar to that of the wild-type: after a 5–10 min incubation at 16 °C, the variant was complexed with hsp70$_{mit}$ in a large amount similar to that of the wild type. When the temperature was raised to 30 °C, K304E-variant MCAD was also completely transferred from hsp70$_{mit}$ to hsp60 within 10 min, suggesting that its binding to and release from hsp70$_{mit}$ and transfer to hsp60 were normal. Thereafter, however, the amount of the variant MCAD bound to hsp60 was considerably greater than that of the wild-type for an extended period of time as we have previously demonstrated.

FIG. 5. Gel filtration of newly imported K304E MCAD in solubilized mitochondria incubated with either apyrase or ATP. In vitro translation product containing K304E pMCAD was incubated with rat liver mitochondria at 30 °C for 10 min. Trypsin-treated mitochondria were solubilized and incubated either with 20 unit/ml apyrase or 2.5 mMs ATP (O—O) at 30 °C for 15 min. Subsequent steps for gel filtration and detection of MCAD were identical with those in the legend to Fig. 1.

FIG. 6. Immunoprecipitation of solubilized mitochondria containing wild-type or K304E-variant MCAD with anti-hsp60 antibody. In vitro translation product containing either wild-type or K304E-variant MCAD was incubated with rat liver mitochondria at 30 °C for 10 min. Trypsin-treated mitochondria were suspended in HMS buffer and a small aliquot (one-twentieth) was taken to determine the total amount of MCAD imported. The rest of the sample was divided into two halves. Mitochondria in one half were immediately pelleted and solubilized, and the other half was further incubated at 30 °C for 15 min before solubilization. Solubilized mitochondria were divided into two and were immunoprecipitated either with 5 μl of anti-hsp60 antiserum (hsp60Ab) or 5 μl of normal rabbit serum (NRS). Immunoprecipitated radiolabeled MCAD and hsp60 were detected by autoradiography and immunoblot analysis, respectively. Intensity of both MCAD band on autoradiogram and hsp60 band on immunoblot was measured by a densitometer and was expressed in densitometric units (unit$_{R}$ and unit$_{I}$, respectively). A, SDS-PAGE fluorogram of wild-type and K304E MCADs. B, quantitative determination of MCAD bound to hsp60. The relative amount of MCAD bound to a unit of hsp60 is shown on the ordinate. The value is expressed as the ratio of the intensity of MCAD band (unit$_{R}$) to that of hsp60 (unit$_{I}$).

DISCUSSION

In the previous study, we had demonstrated that in mitochondria newly imported MCAD formed a high molecular weight complex (hM, complex) which acted as the precursor for tetramer (12). This kinetic behavior, together with the molecular size of the hM, complex and the consideration for the role of hsp60 in the folding and assembly of mitochondrial proteins in general, strongly suggested that the hM, complex represented a complex of MCAD and hsp60. In the present report, we have demonstrated using immunological and biochemical methods that this was indeed the case. Sixty percent of the wild-type
MCAD in the hM₉ complex co-immunoprecipitated with hsp60 when treated with anti-hsp60 antibody. Upon exposure to 2.5 mm ATP, a similar quantity of MCAD was released from the complex. These results indicated that at least 60% of the hM₉ complex was the complex of MCAD and hsp60. The exact cause for the incomplete immunoprecipitation of MCAD in the hM₉ complex with anti-hsp60 antibody is currently unknown. One possibility is that the entire hM₉ complex represented the MCAD-hsp60 complex, but the MCAD moiety was lost from some of the complex during a few cycles of washing after gel filtration. Another possibility is that the hM₉ complex fraction included MCAD-containing macromolecular complexes other than the MCAD-hsp60 complex. Other macromolecular complexes may include the aggregates of unfolded MCAD and the complexes with other proteins. Because of the small size of the gel filtration column used in this study, it was difficult to completely resolve the void volume and the hM₉ complex fraction. The hM₉ complex eluted only a single tube after the void volume. The possibility that MCAD in the hM₉ complex fraction contained MCAD aggregates is unlikely, however, since in this experiment the supernatant of the mitochondrial lysate was applied to the gel filtration column.

Recently, there have been accumulating evidences that after import into mitochondria as unfolded peptide, nuclear-coded proteins interact sequentially, first with hsp70₉mt and then transferred to hsp60 before completing the process of folding and assembly (15–17). We have shown in this paper that both hsp70mt and hsp60 were indeed involved in the folding and assembly of MCAD in mitochondria. When treated with anti-hsp70mt antibody, MCAD co-precipitated with hsp70mt only at a low temperature (16 °C) for a relatively short period of time immediately following the import, indicating that MCAD and hsp70mt formed a transient complex in the early step of mitochondrial import. MCAD protein was quickly transferred to hsp60 when the temperature was raised to 30 °C. MCAD-hsp60 complex survived for a relatively longer period of time even at 30 °C. These data suggest that the binding of MCAD to, and its release from, hsp70mt are very fast at physiological temperature as in the case of Mas2p, the larger of the two subunits of the MAS-encoded processing protease (16), and that the folding of MCAD on hsp60 scaffold is the rate-limiting step in the biogenesis pathway of MCAD tetramer.

The present results, we have shown that after a 15-min chase in intact mitochondria or after ATP addition to the mitochondrial lysate, the amount of the wild-type MCAD-hsp60 complex greatly decreased while the quantity of tetramer inversely increased. Since no monomer was detectable in either experiment, this appeared to indicate that the release of the wild-type MCAD from the MCAD-hsp60 complex and its assembly into tetramer were tightly coupled, as if suggesting that hsp60 assists not only folding of loosely folded proteins into appropriate conformation but also assembly of the correctly folded proteins. However, the currently prevailing view in the study of chaperonins including hsp60 and its bacterial counterpart, groEL, is that while in the process of folding, chaperonins protect loosely folded proteins from forming aggregates via hydrophobic interactions (14, 29, 30). Recently, two groups of investigators have demonstrated that in a protein/groEL complex, protein is contained in the central cavity of groEL double heptamer-toroidal rings with one end closed with groES (31, 32). GroES is the bacterial counterpart of hsp10. It has been considered that once the proteins are correctly folded, monomers are released and they can be automatically assembled without assistance of chaperonin (14). In fact, Zheng et al. (33) have recently shown that upon addition of groES and ATP to the reconstituted ornithine transcarbamylase-groEL complex, ornithine transcarbamylase monomers were first released within 30 s, and, after 90 s, approximately 40% were assembled into the native trimer. At 20 min, no monomers were remaining. In our present experiments, the analysis of the product was done 15 min after chase or after ATP addition. Therefore, it is possible that our observation of the apparent tight coupling of the release of MCAD from its complex with hsp60 and the tetramer assembly could have been the result of a quick sequence of release of monomers, followed by efficient assembly of the resulting monomers into tetramer. It is unknown at present, however, whether or not the behavior of the in vitro reconstituted ornithine transcarbamylase-groEL complex exactly mimics that of the native mammalian enzyme-hsp60 complex in mitochondria. For instance, Viitanen et al. (25) suggested that unlike groEL which is a double heptamer-toroidal ring, mammalian mitochondrial hsp60 is a single heptamer-toroidal ring. Also, MCAD may behave differently from the way ornithine transcarbamylase did in the reconstituted system. There has so far been no conclusive evidence for or against the role of chaperonin in the assembly of correctly folded protein. Thus, the mechanisms of MCAD transfer from the complex with hsp60 to the final tetramer needs to be studied further. In this regard, the findings from the study of K304E-variant MCAD provides important information concerning the mechanisms for the release of appropriately folded MCAD protein from the complex with hsp60 and subsequent assembly into active tetramer as discussed below.

We had previously shown that overall folding/assembly process of the K304E-variant protein was severely impaired, probably at the transfer of the variant MCAD moiety from the hM₉ complex to the tetramer (12). However, the precise mechanism for the impairment was unknown, as the nature of the hM₉ complex and the detailed mechanism of the folding and assembly of MCAD were yet to be studied at the time. In the present paper, we have shown that following the import into mitochondria, the binding of K304E-variant protein to hsp70mt and its transfer to hsp60 were normal. However, the stability of the complex of the variant with hsp60 was markedly different from that of the wild-type. The accurate quantitation of the amount of the wild-type MCAD associated with hsp60 in the hM₉ complex and that of K304E-variant using anti-hsp60 antibody revealed that after a 15-min chase period, only 2% of the wild-type MCAD was remaining in the complex, whereas 44% of the variant MCAD was still found with the complex. Likewise,
when the mitochondrial lysate was incubated with ATP, 21% of the wild-type MCAD was found with the hM\(_{c}\) complex, whereas 64% of K304E still remained with the hM\(_{c}\) complex. These data indicated that the rate of release of the variant from the complex was severely retarded. K304E involves the change of the charge in the interface in the dimer-dimer interaction, not that of monomer-monomer interaction. Since there were no gross accumulation of dimer, an impairment in the assembly of properly folded variant protein into tetramer is unlikely as the cause of the stable nature of the hM\(_{c}\) complex of the K304E-variant. Such a stable nature of the variant complex is likely to be caused by a hindrance in the folding of the variant protein into a proper monomeric conformation while bound to hsp60.

Our recent study on the MCAD biogenesis in riboflavin deficiency provides a clue which is relevant in this regard. We have previously shown, a small amount of tetramer of the wild-type MCAD, which is a large and branched chain a-ketoacid dehydrogenase. El is a hetero-multimeric complex of mamma-

In vitro mixing of individually expressed E1\(_{a}\) and E1\(_{b}\) in Escherichia coli produced biologically active E1, in vitro mixing of individu-

The nature of the wild-type and K304E-variant monomers detected after a 10-min import needs to be discussed. In the folding/assembly pathway of MCAD in the mitochondria, three possible types of monomer can be theoretically predicted. These are loosely folded monomer, which is released from hsp70\(_{\text{nat}}\) before making a complex with hsp60, properly folded monomer released from hsp60, and aged monomer dissociated from the tetramer. It is unlikely that the wild-type and K304E monomers observed at a 10-min import represents either of the latter two types, since no monomer was detected at other time points nor under any other experimental conditions, such as exposure to ATP. Hence, it is likely that the monomers of the wild-type and those of K304E, detected after 10 min of import, may represent the loosely folded monomeric subunits that were released from hsp70\(_{\text{nat}}\) and overflowed the binding capacity of hsp60 as the amount of imported monomer increased from 5 min to 10 min. This hypothesis is further supported by the finding that the amount of the K304E monomer and that in hM\(_{c}\) complex after 10-min import are both considerably greater than the wild-type counterparts.

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