Mitochondrial Import and Processing of Wild Type and Type III Mutant Isovaleryl-CoA Dehydrogenase

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Isovaleric acidemia is a rare inborn error of metabolism caused by a deficiency of isovaleryl-CoA dehydrogenase (IVD), a nucleus-encoded, homotetrameric, mitochondrial flavoenzyme that catalyzes the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA. We have previously identified a nucleotide deletion in the gene for IVD in fibroblasts from a patient with isovaleric acidemia leading to a shift in reading frame and premature termination of translation. The mutant IVD precursor is imported and processed to mature size, but no active enzyme is detected in mutant fibroblasts or expressed in Escherichia coli. Examination of the crystal structure of human IVD reveals that the C terminus is involved in tetramer stability. In vitro mitochondrial import experiments show that wild type IVD protein rapidly and stably forms mature homotetramer following import, whereas Type III mutant protein never forms stable oligomers. An additional series of mutant proteins with truncations and/or alterations in the C-terminal sequence implicates the C terminus of IVD in both enzyme activity and tetramer stability. Importantly, a dimeric intermediate in the folding pathway for wild type IVD has been identified in the in vitro mitochondrial import experiments, the first report of such an intermediate in the biogenesis of an acyl-CoA dehydrogenase.

Isovaleryl-CoA dehydrogenase (IVD)\(^1\) (EC 1.3.99.10) is a mitochondrial flavoenzyme that catalyzes the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA, the third step in the leucine catabolism pathway. The mature enzyme is a homotetramer with a molecular mass of 175 kDa that contains one FAD prosthetic group per subunit (1). IVD is synthesized in the cytosol as a precursor that is 2 kDa larger than the mature subunit. The monomer is translocated into mitochondria, where an N-terminal leader sequence is proteolytically removed followed by formation of mature tetramers (2). Noncovalently bound FAD associates with the apoenzyme, probably after import into the mitochondrion. The lengths of the precursor protein and mature sequence are 424 and 394 amino acid residues, respectively (3). The gene for IVD has been mapped to human chromosome 15 (4) and has been shown to span 15 kilobases, consisting of 12 exons and 11 introns (5). Isovaleryl-CoA dehydrogenase is a member of the acyl-CoA dehydrogenase gene family (3). Different members of this evolutionarily conserved family share 30–35% sequence homology within a species (6), whereas the same gene exhibits 85–90% interspecies sequence identity (3). Despite the high level of conservation among the ACDs, each shows a distinct pattern of substrate utilization, likely due to specific differences in the substrate binding pockets (7, 8).

IVD has been purified to homogeneity from a number of sources, and the crystal structure for recombinant human IVD produced in Escherichia coli has been determined to a resolution of 2.6 Å (7). The native protein is a homotetramer consisting of a dimer of dimers, and its structure is highly conserves with that of the other ACD structures that have been determined (7, 9–11). The opening of the substrate binding pocket is exposed to solvent and contains the adenine moiety of substrate, whereas the isovaleryl carbon backbone is buried in a hydrophobic pocket. The FAD has an extended conformation and is located between the middle and C-terminal domains of one monomer and the C-terminal domain of a neighboring monomer (7). The C terminus of each monomer also overlaps a neighboring subunit.

Isovaleric acidemia is caused by a deficiency of IVD (12). Five different classes of mutations, described on the basis of alterations in size and mitochondrial import of precursor protein, have been identified in cell lines from patients with isovaleric acidemia (13). The Type III mutation, identified in a single patient, is caused by a single base pair deletion at position 1179, leading to a shift in the protein reading frame and resulting in the incorporation of eight abnormally placed amino acids followed by premature termination of translation (14). The mutant monomer is 21 amino acids shorter (2 kDa smaller) than wild type but is efficiently imported into mitochondria in vivo. Fibroblasts expressing this mutation have no detectable IVD activity. To better characterize the role of the C terminus in IVD import, assembly, and catalytic function and the effect of the Type III mutation on these processes, wild type and Type III mutant IVDs were analyzed for their ability to be imported into mitochondria and assembled in vitro. Additional mutant IVDs with smaller C-terminal deletions and substitutions were also constructed in vitro and were similarly analyzed.

EXPERIMENTAL PROCEDURES

Growth of Bacterial Cultures—All bacterial cultures were grown in Luria-Burtoni broth containing 80 \(\mu\)g/ml ampicillin.

Plasmid Construction—For in vitro transcription/translation experiments, the IVD cDNA was subcloned into the Xhol/SpeI sites of a pBluescript KS(-)/(+)-phagemid (Strategene, La Jolla, CA) under control of the T3 or SP6 DNA polymerase promoter. For E. coli expression of IVD, mutations were introduced into a modified pKK223–3 vector (Amerchlam Pharmacia Biotech) containing the wild type IVD gene. The 5’ end of the IVD gene has been modified for optimal E. coli codon bias (15).

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Polymerase Chain Reaction Based Mutagenesis—Polymerase chain reaction (PCR) primers for mutagenesis were synthesized by the Mayo Clinic Molecular Biology Core Facility. Mismatched primers were designed to introduce the desired mutations at the 3′ end of the IVD cDNA (Table I). A 242-nucleotide fragment of the IVD coding region (residues 938-1182) was amplified using bacteria IVD cDNA in Phusion HS Kpf/(-) phageden (Stratagene) as template, leaving a StuI restriction site on the 5′ end and either a KpnI, HindIII, or SpeI restriction site on the 3′ end of the PCR fragment for subsequent subcloning. PCRs were performed either with PCR Supermix (Life Technologies, Inc.) or with a custom mix of reagents (Perkin-Elmer) added to the following final concentrations: 0.2 μM each primer, 2.5 μl of 20× Platinum Taq polymerase, 5 mM MgCl₂, 200 μM each of dATP, dCTP, dGTP, dTTP, in 10 mM Tris/HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3. The reaction was carried out for 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min followed by a final extension at 72°C for 7 min. Polymerase chain reaction products were subcloned into appropriate sites in wild type IVD in either pSP64 poly(A) vector (Promega Corp., Madison, WI) (for in vitro analysis of mitochondrial import) or pKKHIVD (for protein expression and enzymatic analysis). The sequence of all amplified, subcloned products was verified either manually with the 7-deaza-dGTP Sequenase version 2.0 kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions or by automated sequencing by the Mayo Clinic Molecular Biology Core Facility. Sequencing primers for pSP64 and pKKHIVD were synthesized by the Mayo Clinic Oligonucleotide Core Facility and are shown in Table I.

Expression and Purification of Wild Type and Mutant IVDs in E. coli— Cultures of E. coli strain XL-1 Blue (Strategene) with the IVD-containing plasmid (PKK233-3) were grown to mid-log phase, induced with isopropyl-1-thio-β-D-galactopyranoside (final concentration, 1 mM), and incubated with shaking at 37°C overnight. A 1.5-ml culture was harvested (5 min at 9500 × g), washed twice in 750 μl of phosphate-buffered saline, pH 7.4, treated with lysozyme (Roche Molecular Biochemicals), and then subjected to sonication in 25 mM Tris, 1.0 mM EDTA, pH 8.0. Following centrifugation at 16,000 × g for 30 min, IVD activity in the supernatant was quantitated using the anaerobic ETF fluorescence reduction assay as described previously (16, 17). One unit of activity is defined as the amount of enzyme necessary to completely reduce 1 μmol of ETF in 1 min. IVD protein was separated by SDS-PAGE and detected by Western blotting as described previously (18).

In Vitro Mitochondrial Import and Stability of Wild Type and Mutant IVDs— Mitochondria for import experiments were freshly isolated just prior to use from a male Harlan Sprague-Dawley rat liver as described previously (18). Mitochondria for import experiments were freshly isolated just prior to use from a male Harlan Sprague-Dawley rat liver as described previously. IVDs— PAGE and detected by Western blotting as described previously (18).

RESULTS

Expression and Characterization of the Type III Mutant Protein—Extracts from Type III mutant fibroblasts showed no detectable activity as determined by the anaerobic ETF fluorescence reduction assay, whereas extracts from control fibroblasts contained 4.1 ± 0.6 (n = 3) nmol of ETF·min⁻¹·mg⁻¹ cellular protein. A mutant IVD cDNA corresponding to the Type III mutation was constructed by PCR mutagenesis, and molecular modeling was performed on a Silicon Graphics O2 workstation (Mountain View, CA) using the published crystal structures of human IVD and porcine medium chain acyl-CoA dehydrogenase (MCAD) (7, 11) and the Insight II 98 software package (Biosym Technologies, San Diego, CA). The C-terminal amino acids of human MCAD were substituted into IVD, and the predicted structure energy was minimized using the Homology module of Insight II. The final rotamer conformations of the altered amino acids were selected both by minimizing the calculated free energy of each residue and by manual inspection using the Biopolymer module.

Mitochondrial Import and Processing of IVD

Mitochondrial Import, Intramitochondrial Stability, and Assembly of Wild Type and Type III IVD—The E. coli studies suggest that the Type III mutant IVD is unstable following expression. To explore this in the more physiologic milieu of the mitochondria. For these experiments, wild type and Type III IVD proteins were prepared as above but in a 3-fold larger reaction volume. Following import (3 or 30 min), mitochondria were treated with trypsin and one half of the mixture was further incubated in buffer only for either 17 min (for the 3-min pulse) or 90 min (for the 30-min pulse). Mitochondrial matrix contents were isolated as above and applied to a Superdex-200 gel filtration column (BX 16/100, 120 ml bed volume (Amersham Pharmacia Biotech)) equilibrated with 200 mM NaCl, 10 mM HEPES, pH 8.0. Protein migration through the column was calibrated using ribonuclease A (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (668 kDa), and blue dextran (2,000 kDa). Sample was eluted with 200 mM NaCl, 10 mM Tris-Cl, pH 8.0, and 1.5-ml fractions were collected. IVD protein was immunoprecipitated, denatured, and visualized as described above.

Molecular Modeling of IVD Mutations—Molecular modeling was performed on a Silicon Graphics O2 workstation (Mountain View, CA) using the published crystal structures of human IVD and porcine medium chain acyl-CoA dehydrogenase (MCAD) (7, 11) and the Insight II 98 software package (Biosym Technologies, San Diego, CA). The C-terminal amino acids of human MCAD were substituted into IVD, and the predicted structure energy was minimized using the Homology module of Insight II. The final rotamer conformations of the altered amino acids were selected both by minimizing the calculated free energy of each residue and by manual inspection using the Biopolymer module.

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FIG. 1. Western blot of wild type and Type III IVD expressed in E. coli. Cells containing either a wild type or Type III IVD expression vector were induced with isopropyl-1-thio-β-D-galactopyranoside for 30 min, 4 h, or 24 h at 37°C. One ml of cells was collected by centrifugation at each time point and resuspended in 100 μl of SDS-sample buffer. Twenty μl were subjected to electrophoresis by SDS-PAGE, the gel was transferred to polyvinylidene difluoride membrane, and IVD was visualized with an anti-IVD antiserum. Lanes 1, 3, 5, and 7, wild type IVD, 0 min, 30 min, 4 h, and 24 h, respectively, after induction. Lanes 2, 4, 6, and 8, Type III IVD, 0 min, 30 min, 4 h, and 24 h, respectively, after induction. Lanes 9 and 10, wild type and Type III IVD cultures after 24 h growth without induction. WT, wild type; III, Type III mutant; Nil, not induced.
Mitochondrial Import and Processing of IVD

**FIG. 2. Stability of wild type and Type III mutant IVD following in vitro import into mitochondria.** Wild type and type III IVD cDNA were transcribed and translated in vitro and incubated with isolated rat liver mitochondria for 30 min (pulse). The mitochondria were then washed in buffer, trypsin-treated, and further incubated at 30°C for an additional 30, 90, or 210 min. At the indicated times, an aliquot of mitochondria was removed, and the matrix contents were isolated and immunoprecipitated with anti-IVD antiserum. The immunoprecipitants were boiled in SDS sample buffer, separated by SDS-PAGE, and visualized on a PhosphorImager, and bands corresponding to IVD were quantitated using Imagequant software (Molecular Dynamics). A, 12% SDS-polyacrylamide gel of IVD translation and mitochondrial import products. The top panel shows results with wild type IVD, and the bottom panel shows results with the Type III mutant. Lane 1, translated IVDs prior to mitochondrial import (Tr); lane 2, IVDs imported into mitochondria in vitro for 30 min (pulse (Pu)). Lanes 3, 4 and 5, imported IVDs 30, 90, and 210 min, respectively, after removal of IVD from the import reaction. The migration position of precursor and mature IVDs are indicated by P and M, respectively. B, percentage of IVD protein remaining within the mitochondrial matrix after the indicated period of incubation as compared with the amount of imported subunits detectable after a 30-min pulse without incubation. *T*1/2, the time calculated for half of the newly imported IVD subunits to be degraded, is 94 min for the wild type protein and 41 min for the Type III mutant. ■, wild type IVD; ●, Type III mutant IVD.

mitochondrial matrix, an in vitro mitochondrial import assay was utilized. Translated precursor wild type and Type III IVD were incubated with mitochondria, and the amount of mature-size subunits accumulating in the mitochondrial matrix after 30 min of import was visualized via SDS-PAGE. Both the Type III and wild type IVDs were imported and processed to a mature form with similar efficiencies in this system (Fig. 2A, lane 2). To evaluate the stability of the imported subunits, the precursor protein was removed by trypsinization and extensive washing, and the amount of IVD signal remaining in the matrix at various times after import was measured via SDS-PAGE. Both the Type III and wild type IVDs were imported and processed to a mature form with similar efficiencies in this system (Fig. 2A, lane 2). After 30 min, about 75% of the imported wild type protein remained in the matrix, with a *t*1/2 for loss of the signal of 94 min (Fig. 2B). In contrast, half of the signal from imported Type III IVD disappeared from the matrix in about 40 min.

Gel filtration studies of mitochondrial matrix contents were performed at various time points after subunit import to characterize the quaternary structure of the imported IVD subunits. The elution profiles from the gel filtration column are shown in Fig. 3. After a 3-min pulse, most of the wild type IVD signal was recovered in fractions corresponding to a molecular size of about 80 kDa (Fig. 3A). After an additional 17-min incubation with buffer alone, more signal was detected in these fractions; however, a shoulder on this peak corresponding to a higher molecular mass species was also evident. Thirty min after import, most of the material eluted in fractions corresponding to a molecular mass of about 170 kDa, the predicted size of wild type IVD tetramer. Wild type IVD that had been incubated with mitochondria for 30 min and then incubated for 90 min without IVD precursor was found primarily in the fractions corresponding to 170 kDa. Fig. 3B shows the elution profiles of the Type III mutant protein. At all time points, the import signal was scattered over the entire elution volume. After a short pulse, most of the counts were found in fractions corresponding to smaller molecular weight species, and at later time points, signal was detected in fractions representing higher molecular weight species. A comparison of the percentage of counts from each time point corresponding to a molecular mass between 40 kDa (monomer) and 180 kDa (tetramer) from the wild type and Type III profiles is depicted graphically in the inset of Fig. 3A. For the wild type protein, both the absolute number of counts and percentage of total counts was relatively constant over time (data shown only for percentage of total counts). For the Type III mutant IVD, both the absolute amount and percentage of total counts decreased steadily over time.

**Relationship of the Length of the IVD Monomer C Terminus to Enzyme Function and Stability**—The Type III mutant protein differs from wild type IVD at the C terminus in amino acid sequence (due to the shift in reading frame) and length (due to premature termination of translation). Examination of the x-ray crystal structure of wild type IVD reveals significant subunit interactions involving the C termini of adjacent monomers (7). To further investigate the role of the C terminus in formation and stability of IVD tetramers, IVD mutants with progressively longer C-terminal deletions and/or specific amino acid substitutions were constructed. The mutant IVD cDNAs were then expressed in *E. coli* and examined for enzymatic activity using the ETF fluorescence reduction assay. Relative stability was assessed following in vitro transcription/translation and mitochondrial import. The amino acid sequences and relative stability of these mutants compared with wild type enzyme are detailed in Fig. 4. Only one mutant (DEL1) was active when produced in *E. coli*. With the exception of the DEL1 mutant, all of the deletion mutant enzymes displayed decreased stability compared with wild type IVD following in vitro import into mitochondria (Table I). Stability of the DEL5 mutant protein was 68% that of wild type IVD protein, and progressively longer deletion mutants were increasingly less stable in direct proportion to the deletion length (relative stability as calculated by linear regression using the least squares method = −0.0399 × deletion length + 0.955; *r* = −0.955). Replacement of the last seven amino acids at the C terminus of wild type IVD with either Ala or Gly led to loss of measurable enzyme activity (not shown), but the ALA7 mutant was stable after import into mitochondria in vitro, whereas the GLY7 mutant had reduced stability. A mutant IVD containing the last 10 amino acids of MCAD substituted for the final 9 residues of wild type IVD.
curves), based on molecular mass standards, are shown in each panel. Inset A, radioactive signal was quantitated using a PhosphorImager. The percentage of total counts eluting within each fraction is shown graphed as a curve in both panels correspond to the legend shown in B.

FIG. 3. Mitochondrial import and processing of wild type and type III mutant IVD. IVD protein was prepared by in vitro transcription/translation. Translated protein was incubated with freshly prepared mitochondria for the indicated times (pulse). Mitochondria were washed in buffer, trypsin-digested, and further incubated. Matrix contents were isolated and applied to a Superdex 200 gel filtration column. Fractions of 1.5 ml were collected and immunoprecipitated with IVD antiserum. Samples were boiled in SDS sample buffer and separated via SDS-PAGE. The calculated positions for elution of tetramer (T), dimer (D), and monomer (M), based on molecular mass standards, are shown in each panel. Curves in both panels correspond to the legend shown in B.

Molecular Modeling of the IVD C Terminus—The C terminus of the IVD monomer forms an α-helix, which interacts not only with FAD and substrate but also with helical domains H and I from this face, but the introduction of the extra charged residues brings two pairs of residues in position to interact ionically (Arg-387 with Glu-379 of the same subunit (4.3 Å distance between charged groups). This basic configuration is predicted to be unchanged when the C-terminal amino acids derived from MCAD are substituted for those of IVD (5.3 Å). The charged residues are still predicted to be oriented away from this face, but the introduction of the extra charged residues brings two pairs of residues in position to interact ionically (Arg-387 with Glu-379 of the same subunit (4.3 Å distance between charged groups). The charged surface of this helix, however, is not predicted to interact with another monomer.

DISCUSSION

The Type III IVD mutation leads to a shift in reading frame and the subsequent incorporation of eight abnormally placed amino acids with premature termination of translation. The IVD protein produced by this allele is of reduced molecular size but is imported into mitochondria efficiently. Extracts from patient fibroblasts expressing this mutation were reported to have no IVD activity as measured with a radiolabeled substrate (13). Here, we confirm the lack of activity in extracts from this cell line using the sensitive and specific ETF fluorescence reduction assay utilizing isovaleryl-CoA as substrate. On the molecular level, there are many potential effects of this mutation; functional impairment may result from major structural consequences of the truncation (e.g. failure to fold and/or oligomerize properly) or from a missing critical amino acid residue that plays a specific role in import, processing, or enzymatic function. A combination of expression studies in E. coli and in vitro mitochondrial import studies has been utilized to delineate the functional effects of the Type III mutation on IVD and to characterize the role of the C terminus in IVD subunit tetramerization and tetramer stability.

Wild type IVD protein produced in E. coli was detectable by Western blotting as early as 30 min following induction, and

FIG. 4. Alignment of ACD C termini and IVD C-terminal mutants. Mutant IVDs were constructed by PCR mutagenesis and confirmed by DNA sequencing. The top part of the figure shows an alignment of five human ACDs. The consensus line indicates positions at which the amino acid residue is conserved in four of five or in all five of the ACDs. The bottom portion of the figure shows the C termini of the IVD mutants studied via prokaryotic expression and in vitro mitochondrial import experiments. The relative stability of mutant IVDs compared with wild type enzyme 120 min following in vitro import into mitochondria is shown to the right of each mutant. The amino acid residue numbering is that for mature wild type human IVD; HIVD, human IVD; HMCAD, human short chain acyl-CoA dehydrogenase; HMISCAD, human short chain acyl-CoA dehydrogenase; HLCAD, human long chain acyl-CoA dehydrogenase; HLSBACD, human short, branched chain acyl-CoA dehydrogenase. See text for descriptions of the mutant enzymes.
high levels of protein persisted for 24 h. Type III mutant IVD was also identifiable 30 min and 4 h after induction, but at 24 h, no mutant IVD protein was detectable in either whole cells or cellular extracts. These results suggest that Type III mutant IVD is unstable when produced in *E. coli*, a result of improper folding and/or oligomerization of mutant subunits, instability of mutant tetramers once they are formed, or a combination thereof.

Nucleus-encoded proteins that are active in the mitochondrial matrix are first synthesized in the cytoplasm and then targeted to the mitochondria via a signal peptide, where they are imported following interaction with specific membrane receptor complexes. The signal peptide is cleaved in the matrix followed by final folding and oligomer assembly. *In vitro* mitochondrial import studies with MCAD have shown that subunits imported into mitochondria first associate with the molecular chaperone hsp70 and subsequently with hsp60 in a high molecular weight complex (20–22). This complex disappears, and a molecular species consistent in size with that of mature tetramers is identified. Both the commonly identified K304E mutation and depletion of intramitochondrial FAD lead to impaired release of IVD from the hsp60 complex (20, 22). In the current study, IVD was not found to associate with a high molecular weight complex, even after very short periods of import (Fig. 3) or when mitochondrial import was performed at low temperature to retard the import process (data not shown). Thus, the postimport folding of IVD subunits may be more rapid than described for MCAD. Following *in vitro* mitochondrial import, IVD subunits were identified in the mitochondrial matrix in a form with molecular mass of approximately 90 kDa.
(Fig. 3A), too small to represent folding IVD subunits associated with an hsp70 or 60 as seen for MCAD. Because the overall structure of IVD is a dimer of dimers, it is reasonable to hypothesize that newly imported monomers first form dimers, which subsequently associate into the final tetrameric form. The 90-kDa IVD form identified is of appropriate size to represent a dimeric assembly intermediate, the first such evidence for such a transitional molecule. Additional experiments will be necessary to further characterize the nature of the 90-kDa species.

In vitro mitochondrial import experiments with the Type III mutant IVD indicate that it is translated and imported into mitochondria efficiently, as described previously in cell studies (13). Thus, cytoplasmic folding and mitochondrial targeting and import appear to be unaffected by this mutation. In contrast, the mutant protein disappears from the mitochondrial matrix much more rapidly than wild type enzyme (Fig. 2). Structural data reveal that the C termini of the IVD subunits are important in stabilization of the tetramer through monomer-monomer interactions (7). Therefore, it seems likely that the abnormal C terminus of Type III mutant IVD leads to decreased tetramer assembly and/or stability. In agreement with this, gel filtration studies of Type III mutant protein show no evidence of formation of mature mutant tetramers following mitochondrial import.

The Type III IVD mutant subunit is shorter than wild type and contains an abnormal amino acid sequence at the C terminus. To isolate the role of these factors in IVD assembly and activity, site-specific in vitro mutagenesis was used to prepare a number of mutant IVDs altered at the C terminus (Fig. 4). Deletion of a single residue at the C terminus did not affect enzyme activity; however, a five-residue deletion led to the complete loss of detectable IVD activity in crude E. coli extracts following overexpression of the mutant IVD. This inactive mutant was partially stable following import into mitochondria. Additional truncated proteins missing 10, 13, 16, or 19 residues at the C terminus had no detectable enzyme activity when produced in E. coli and showed a linear inverse relationship between chain length and stability. The crystal structure of human IVD demonstrates that residues with neutral polar or nonbulky side chains in this region predominately interact with the opposing monomer. Removal of these residues will reduce the number of subunit contacts available and would be expected to reduce tetramer stability as seen. Replacement of seven C-terminal residues with alanine led to a protein that was stable in mitochondria but inactive when produced in E. coli, whereas a mutant IVD with glycines substituted for the final seven C-terminal amino acids was neither active nor expected to reduce tetramer stability as seen. Replacement of these residues is predicted to be in a position to interact with Arg-387, potentially decreasing its ability to react with substrate and resulting in an inactive enzyme. The increased stability of this mutant protein is most likely related to subtle effects on monomer conformation of the additional ionic interactions predicted to occur at the C terminus (Arg-387 with Glu-388, and Glu-391 with either Lys-392 or Lys-394), because the side chains of these residues, along with that of Lys-394, face away from the interacting monomer.

In summary, these studies provide evidence for a major role of the C terminus of IVD in subunit interaction and tetramer stability. The primary effect of the Type III patient and other C terminus mutations is likely an inability of mutant subunits to form stable tetramers rather than a defect in subunit folding itself. A novel dimeric intermediate in IVD tetramer formation within the mitochondrial matrix has also been identified. Study of additional mutant IVDs will continue to provide insights into the important process of assembly of this mitochondrial matrix protein.

REFERENCES

1. Ikeda, Y., and Tanaka, K. (1983) J. Biol. Chem. 258, 1077–1085
2. Ikeda, Y., Keese, S., Fenton, W. A., and Tanaka, K. (1987) Arch. Biochem. Biophys. 252, 662–674
3. Matsubara, Y., Ikeda, Y., Naito, E., Ozasa, H., Glassberg, R., Vockley, J., Ikeda, Y., Kraus, J., and Tanaka, K. (1989) J. Biol. Chem. 264, 16321–16333
4. Kraus, J. P., Matsubara, Y., Barton, D., Yang-Feng, T. L., Glassberg, R., Ito, M., Ikeda, Y., Mole, J., Francke, U., and Tanaka, K. (1987) Genomics 1, 264–269
5. Parimoo, B., and Tanaka, K. (1993) Genomics 15, 582–590
6. Ikeda, Y., Yang-Feng, T., Glassberg, R., and Tanaka, K. (1995) Genomics 11, 609–620
7. Tiffany, K. A., Roberts, D. L., Wang, M., Paschke, R., Moshen, A. W. A., Vockley, J., and Kim, J. J. P. (1997) Biochemistry 36, 8455–8464
8. Kim, J.-J. P., Wang, M., Paschke, R., Djordjevic, S., Bennett, D. W., and Vockley, J. (1994) in Plasmin and Plasminogenas 1993 (Yagi, K., ed) pp. 273–282, Walter deGruyter, New York
9. Battaille, K. P., Moshen, A.-W., and Vockley, J. (1996) Biochemistry 35, 15356–15363
10. Djordjevic, S., Pace, C. P., Stankovich, M. T., and Kim, J. J. P. (1995) Biochemistry 34, 2163–2171
11. Kim, J. J. P., Wang, M., and Paschke, R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7523–7527
12. Tanaka, K., Budd, M. A., Efron, M. L., and Iselbacher, K. J. (1966) Proc. Natl. Acad. Sci. U. S. A. 56, 236–242
13. Ikeda, Y., Keese, S., and Tanaka, K. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7081–7085
14. Vockley, J., Parimoo, B., and Tanaka, K. (1991) Am. J. Hum. Genet. 40, 147–157
15. Moshen, A.-W., and Vockley, J. (1995) Gene 160, 263–267
16. Moshen, A.-W., and Vockley, J. (1995) Biochemistry 34, 10146–10152
17. Beckmann, J. D., Freeman, F. E., and McKean, M. C. (1981) Biochem. Biophys. Res. Commun. 102, 1290–1294
18. Moshen, A.-W., Anderson, B., Volchenboum, S., Battaille, K., Tiffany, K., Roberts, D., Kim, J.-J., and Vockley, J. (1998) Biochemistry 37, 10325–10335
19. Losmih, U. K. (1979) Nature 227, 680–685
20. Saito, T., and Tanaka, K. (1995) J. Biol. Chem. 270, 1899–1907
21. Yokota, I., Saito, T., Vockley, J., and Tanaka, K. (1992) J. Biol. Chem. 267, 26904–26910
22. Saito, T., Welch, W. J., and Tanaka, K. (1994) J. Biol. Chem. 269, 4401–4408

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