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Characterization of Wild-Type Human Medium-Chain Acyl-CoA Dehydrogenase (MCAD) and Mutant Enzymes Present in MCAD-Deficient Patients by Two-Dimensional Gel Electrophoresis: Evidence for Post-translational Modification of the Enzyme

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Two-dimensional gel electrophoresis was used to study and compare wild-type medium-chain acyl-CoA dehydrogenase (MCAD; EC 1.3.99.3) and missense mutant enzyme found in patients with MCAD deficiency. By comparing the patterns for wild-type and mutant MCAD expressed in *Escherichia coli* or in eukaryotic COS-7 cells we demonstrate that variants with point mutations changing the net charge of the protein can be readily resolved from the wild-type protein. After expression of the cDNA in eukaryotic cells two spots representing mature MCAD can be distinguished, one with an isoelectric point (p*I*) corresponding to that obtained for the mature protein expressed in *E. coli* and another one shifted to lower p*I*. This demonstrates that MCAD protein is partially modified after transport into the mitochondria and removal of the transit peptide. The observed p*I* shift would be compatible with phosphorylation of one aspartic acid residue per monomer. Comparison of pulse labeling and steady-state amounts of MCAD protein in overexpressing COS-7 cells confirms that K304E MCAD is synthesized and transported into mitochondria in amounts similar to the wild-type protein, but is degraded much more readily. For wild-type MCAD, the spot representing the nonmodified form predominates after pulse labeling while that representing the modified form is relatively stronger in steady state, demonstrating that the modification occurs in mitochondria after the transit peptide has been removed. For K304E mutant MCAD, the nonmodified spot is relatively stronger both in pulse labeling and in steady state, indicating that either the efficiency of modification or the stability of the modified form is affected by the K304E mutation. Detection of both wild-type and K304E mutant MCAD was achieved in lymphoblastoid cells from patients and carriers of the mutation. Both spots for the wild-type but only the nonmodified spot for the K304E mutant could be detected. In lymphoblastoid cells from carriers, the intensity of the spot representing the mutant protein is much weaker than the two spots representing wild-type MCAD, emphasizing that the K304E mutant protein is more susceptible to degradation than wild-type MCAD. The absence of detectable amounts of modified K304E mutant MCAD protein in these cells suggest that the conclusion drawn from COS-7 cell expression is also valid in patient cells.

Medium-chain acyl-CoA dehydrogenase (MCAD; EC 1.3.99.3) is one of four chain-length-specific enzymes which catalyze the initial α,β-dehydrogenation step in mitochondrial β-oxidation of straight-chain fatty acids (1,2). MCAD is a tetrameric flavoprotein with a subunit molecular weight of approximately 43600 Da for the mature monomer. It is synthesized as a preprotein (pre-MCAD) in the cytoplasm and subsequently transported into the mitochondria where it is processed proteolytically

and folds to the native enzyme. The cDNA sequence and the organization of the gene have been determined (3,4) and the three-dimensional structure of pig liver MCAD is available at 2.4 Å resolution (5).

MCAD deficiency, a recessively inherited disease due to mutations in the MCAD gene, has received considerable attention in the recent past due to its high incidence and potentially fatal outcome (6,7). Stimulated by the publication of the cDNA sequence, the molecular basis of the disease has been investigated in detail. One prevalent point mutation (G985) which leads to the substitution of glutamic acid for lysine at position 304 (K304E) of the mature polypeptide has been identified (8–11). It is present in about 90% of the alleles of patients (12) and its carrier frequency in Northern European Caucasians is as high as 1 in 40 to 1 in 100 resulting in an expected homozygote frequency of 1 in 6400 to 1 in 40,000 (13). It has been demonstrated that MCAD protein with the K304E substitution is transported into mitochondria with an efficiency similar to that of the wild-type, but that it is much more susceptible to degradation (14,15,16). By heterologous expression in *Escherichia coli,* we have shown that the mutant protein is impaired in folding and probably also oligomer assembly, but that a small proportion of the protein acquires the native structure and displays enzyme activity in the range of the wild-type protein (17). Together with the results from *in vitro* translation experiments (18), this suggests that the accumulation of labile folding intermediates might be the reason for a higher susceptibility to degradation of K304E MCAD. Several rare mutations (a series of point mutations, a 4-bp deletion and a 13-bp insertion) have also been characterized as disease-causing in patients (12,19,20). In most of the cases, these mutations are present in compound heterozygosity with G985 (K304E).

Of the eight missense mutations in MCAD deficiency known until now, five alter the net charge of the polypeptide making it possible to separate the variants on basis of their isoelectric points. Horizontal two-dimensional gel electrophoresis, using an immobilized pH gradient in the first dimension, has been shown to yield very reliable patterns which can be standardized easily (21).

The objective of this study was to set up two-dimensional gel electrophoretic characterization for wild-type and mutant MCAD proteins in patient material in order to be able to characterize both MCAD variants in compound heterozygous cells and in carriers. Using heterologous *E. coli* and COS-7 cell-derived expression systems for the assignment of the spots of wild-type and K304E MCAD, we discovered that MCAD protein is partially modified in eukaryotic cells. Modification of wild-type and K304E mutant MCAD was analyzed in overexpressing COS-7 cells and lymphoblasts. We suggest that a decreased rate of modification or an increased rate of degradation of the modified K304E mutant enzyme is implicated in the disease mechanism.

MATERIALS AND METHODS

2D gel electrophoresis. Two-dimensional gel electrophoresis was carried out with a Multiphor apparatus (Pharmacia). Isoelectric focusing in the first dimension was performed on an immobilized pH gradient (Immobiline dry strips; Pharmacia) with a pH range from 3.0 to 10.5 essentially as recommended by the supplier. After the first dimension, the strips were equilibrated for 2 × 10 min in equilibration solution (100 mM Tris–Cl, pH 6.8; 6 M urea; 25.5% glycerol) supplemented with 16 μM DTT in the first and 0.25 mM iodoacteamide in the second incubation, respectively. The equilibrated strips were either subjected to the second dimension directly or stored frozen at −20°C. The second dimension was carried out in horizontal SDS–PAGE with a discontinuous buffer system. Gels (195 × 250 × 0.5 mm) were cast on GelBond PAG film (Pharmacia). The composition of the gels was as follows—resolving gel: 10% T, 3.3% C, 0.1% SDS, 375 mM Tris–Cl, pH 8.8; stacking gel: 5% T, 3.3% C, 0.1% SDS, 120 mM Tris–Cl, pH 6.8. Two equilibrated strips were placed directly on the cathodic side of the second dimension gel. Electrophoresis conditions were (maximum settings) 50 min at 600 V, 20 mA, and 30 W, then the strips were removed and after 10 min at 600 V, 50 mA, and 30 W the cathodic wick was moved forward so that it overlapped the former application area of the strips and electrophoresis was continued for 2–2¼h at 600 V, 50 mA, and 30 W until the bromphenol blue reached the anodic wicks. The gels were either stained with silver according to Heukeshoven and Dernick (22) or subjected to Western blotting. For blotting, the gel was cut in the middle and the support film removed with a film remover (Pharmacia). Both halves of the gel were blotted onto Immobilon membranes (Millipore) using a semidry apparatus (NovaBlot; Pharmacia) and developed immunologically as described by Blake *et al.* (23) using polyclonal anti-MCAD antibodies (24) and alkaline

phophatase-conjugated secondary antibodies. Staining either for color detection with NBT/BCIP or for chemiluminescence detection with AMPPD (Tropix) was performed in glycine buffer (100 mM glycine; 1 mM $MgCl_2$; 1 mM $ZnCl_2$; pH 9.6). In some cases, a colloidal gold (BioRad) total protein stain was applied after the immunostaining procedure as recommended by the supplier. For autoradiography, gels were fixed (40% ethanol, 10% acetic acid) for 30 min and then incubated 2 × 1 h in dimethyl sulfoxide and finally 1 h in dimethy sulfoxide with 22% (w/v) 2,5-diphenyloxazole (PPO). The gel was washed extensively with cold tap water, incubated 30 min in 10% glycerol, and air-dried under a layer of cellophane. The dried gel was exposed to X-ray film.

Heterologous expression in E. coli and COS-7 cells. Expression of wild-type and K304E mutant MCAD in *E. coli* was performed as described using the pBluescriptKS$^-$(Stratagene)-derived plasmids encoding the mature part of wild-type and K304E MCAD (17). For expression of R28C MCAD, PCR was performed using a cDNA clone carrying the T157 mutation (19) with the mutagenic sense strand primer introducing a *Nde*I site with an ATG start codon directly 5′ to the first codon of the mature protein (24) and a primer complementary to the MCAD sense strand. The PCR product was digested with *Nde*I and *Pst*I and the fragment encoding the first 40 amino acids of mature MCAD plus the initiator methionine was introduced into the plasmid encoding wild-type MCAD (pBMCK2$^-$) replacing the respective wild-type sequence. Cells were grown at 37°C, induced with 1 mM isopropyl-β-D-thiogalactopyranoside at an OD_{600} of 0.7–0.9, grown for 14 additional hours, and harvested by centrifugation. COS-7 cells transfected with the plasmids containing the cDNA for wild-type or K304E pre-MCAD were grown and harvested as described by Jensen *et al.* (16).

Growth of primary human fibroblasts and lymphoblastoid cells. Primary human fibroblasts and lymphoblastoid cells obtained from Epstein–Barr virus-transfected primary lymphocytes were grown using RPMI 1640 supplemented with 10% fetal calf serum.

Preparation of samples. Cell pellets were solubilized in lysis buffer (9 M urea, 2% Triton X-100, 260 mM 2-mercaptoethanol, 2% Pharmalyte 3.5–10.0, 1 mM phenylmethylsulfonyl fluoride) to a final protein concentration of 2–10 μg/μl for *E. coli,* COS-7 cells and lymphoblastoid cells and 5–20 μg/μl for primary human fibroblasts. Samples were used directly or stored at −20°C. Before electrophoresis, 1- to 5-μl aliquots from the cell pellets solubilized in lysis buffer were diluted at least 1:5 in sample solution (8 M urea, 260 mM 2-mercaptoethanol, 2% Pharmalyte 3.5–10.0, 0.5% Triton X-100, 0.01% bromphenol blue).

Metabolic labeling of COS-7 cells with [^{35}S]methionine. This was performed essentially as described in (25). Briefly, cells were labeled in medium containing 0.2 mCi/ml [^{35}S]methionine for 30 min at 37°C. Immediately thereafter, cells were washed in medium containing nonlabeled methionine at a concentration of 150 mg/ml, and washed 2 min with phosphate-buffered saline. The cell pellet was processed and analyzed by two-dimensional gel electrophoresis followed by autoradiography as described above.

RESULTS

2D Gel Electrophoretic Pattern of Wild-Type MCAD and Two Variants Carrying Point Mutations Expressed in E. coli

The isoelectric point (p*I*) of mature wild-type MCAD determined theoretically using the ISOELECTRIC program of the Genetics Computer Group Sequence Analysis Software Package (26) is 7.22. The net charge versus pH curve is flat around the p*I*, predicting that mutations altering the net charge of the polypeptide will lead to observable changes in the isoelectric point.

We have recently described an *E. coli*-based bacterial system for overexpression of the mature part of human MCAD preceeded by an artificially introduced initiator methionine (11,24). This system was used to express mature wild-type, the prevalent disease-causing K304E mutant and a mutant detected in two patients and exchanging arginine-28 with cysteine (R28C; 19). The mutants alter the net charge by one (R28C) or two (K304E) charge units and have theoretically calculated isoelectric points of 6.75 and 6.35, respectively. Total cell pellets were solubilized, subjected to 2D electrophoresis (isoelectric focusing on immobilized pH gradient gels (pH 3.0–10.5) followed by SDS–PAGE in 10% Laemmli gels) and silver stained as described under Materials and Methods. As a control, *E. coli* cells transformed with the vector plasmid were used. Figure 1 shows the 2D pattern of cells expressing wild-type MCAD,

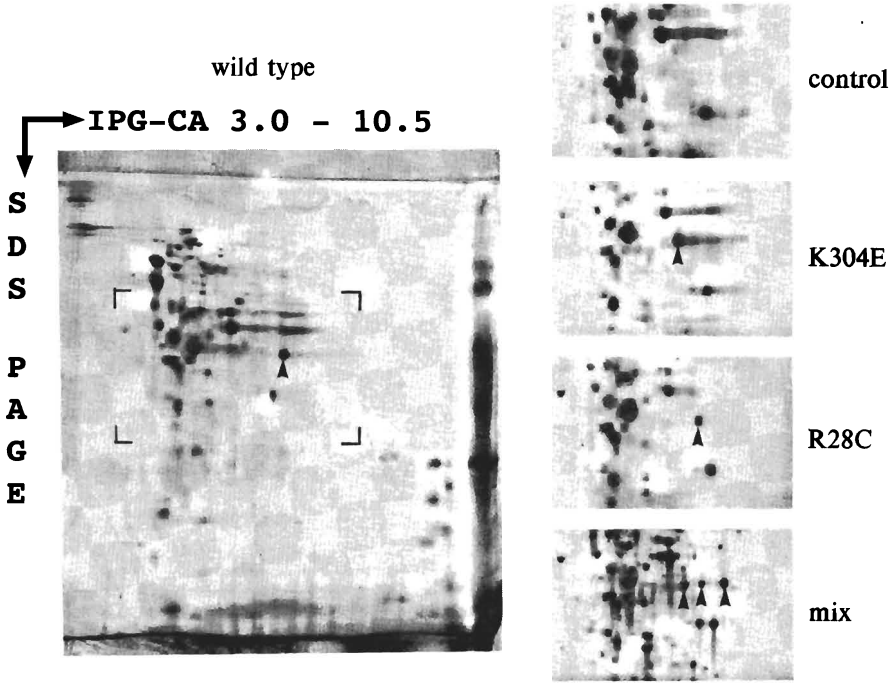

FIG. 1. Two-dimensional gel electrophoresis of *E. coli* cells expressing wild-type or mutant human MCAD. *E. coli* TG1 cells transformed with plasmids encoding the mature form of wild-type, K304E, or R28C MCAD or cells transformed with the vector plasmid (control) were grown and induced as described under Materials and Methods. Aliquots of harvested cells were subjected to two-dimensional gel electrophoresis and the proteins were visualized by silver staining as described under Materials and Methods. The entire gel of cells expressing wild-type MCAD and sections (as indicated) of the gels from cells expressing the variants and the control are shown. The arrowheads indicate the position of the spots for the respective MCAD polypeptides. A mixture (mix) from samples of cells expressing wild-type, K304E, and R28C MCAD (marked by arrowheads from right to left) is shown at the bottom.

sections from the gels with cells expressing K304E or R28C MCAD, and a control. For wild-type, K304E and R28C MCAD one unique spot each can be detected, which is not present in the 2D gel with the control cells. In the gel where the three samples were mixed, the three spots representing wild-type MCAD and the two variants can be distinguished. This demonstrates that the gel system used can resolve one charge differences of MCAD protein. The apparent isoelectric points are in good agreement with the theoretically calculated values.

2D Pattern of COS-7 Cells Overexpressing Wild-Type and Mutant MCAD

In the next step, the location of the MCAD spots for wild-type and the K304E point mutant expressed in eukaryotic cells was analyzed. The COS-7 cell-derived expression system described by Jensen *et al.* (16) was used for this purpose. COS-7 cells transfected with plasmids containing the cDNA encoding either wild-type or K304E pre-MCAD were analyzed. As a control, vector-transfected COS-7 cells were used. The 2D patterns are displayed in Fig. 2. Comparing the silver-stained gels of COS-7 cells overexpressing wild-type MCAD with the control, two MCAD-specific spots with the same molecular weight can be observed for wild-type MCAD. The spot with the more acidic p*I* is more pronounced. For comparison of the location of these spots in relation to the one from mature MCAD expressed in *E. coli,* the *E. coli* and COS-7 cell samples were mixed and electrophoresed collectively. The more basic spot of COS-7 cell-expressed MCAD coincides with the spot representing the mature wild-type protein expressed in *E. coli.* This was documented by overlay of the gel with the mixed samples with gels where the individual samples were electrophoresed. These data suggest that the more acidic spot detected in COS-7 cells represents a modified MCAD protein.

The observed p*I* shift is less than that observed for the R28C mutant expressed in *E. coli.* This mutation causes the exchange of arginine, which is fully protonated at the isoelectric point of wild-type

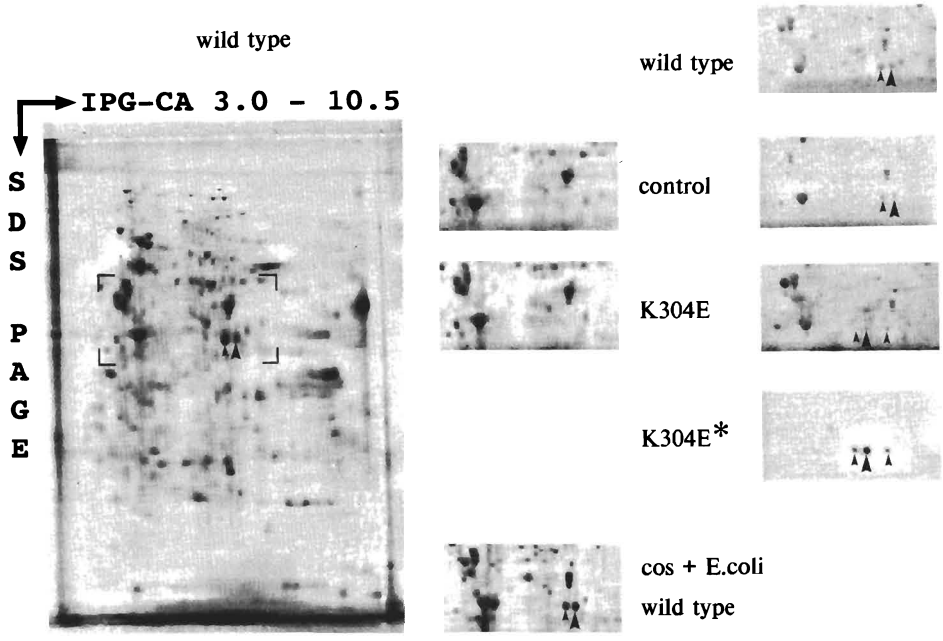

FIG. 2. Two-dimensional electrophoresis of COS-7 cells transfected with a plasmid containing the cDNA encoding wild-type or K304E pre-MCAD, or the vector plasmid. Two-dimensional electrophoresis was carried out as described under Materials and Methods. The entire silver-stained gel from a sample of cells expressing the wild-type gene and sections from gels loaded with the material indicated are shown. Middle panel: silver staining; right panel: immunostaining followed by colloidal gold total protein stain. For the sample designated K304E* immunostaining was performed with chemiluminescence without subsequent colloidal gold staining. The position of the spots for the nonmodified forms are indicated with large arrowheads and those for the modified forms with small arrowheads.

MCAD, with cysteine, which does not carry a charge at this pH, and therefore produces a difference by a full unit in the net charge. The p*I* shift observed for the modified spot must hence be brought about by a group with a p*K* in the range of from 6 to 8 in order to explain the observation.

The more acidic MCAD spot comprising the modified form of MCAD has an apparent p*I* of approximately 6.9. In the human keratinocyte two-dimensional database (27), a p*I* of 6.89 has been determined experimentally for MCAD which is in good agreement with the p*I* for the modified spot and indicates that the modified form predominates in steady-state keratinocytes.

Spots for the endogenous MCAD in COS-7 cells and overexpressed K304E MCAD could not be detected unambiguously by silver staining. Instability of K304E MCAD and a lower yield of the protein in steady state after overexpression in COS-7 cells has been reported previously (16). Therefore, Western blotting after the second dimension SDS–PAGE was performed using anti-MCAD antibodies for detection. In order to be able to overlay the patterns from individual gels correctly a colloidal gold total protein stain of the filter was performed after the immunostaining procedure. The spots stained specifically with the antibody can readily be distinguished from the total protein stain by the different tone of the colors on the original sheets.

There were two spots observed for the endogenous COS-7 cell MCAD which precisely concur with the spots for overexpressed human wild-type MCAD. Also, here the modified form is predominant. In the immunoblot of COS-7 cells expressing K304E-mutant MCAD, three spots can be observed, one which represents the modified form of the endogenous COS-7 cell MCAD and two K304E-specific ones with a lower isoelectric point. Furthermore, the more basic of the latter spots coincides with the K304E MCAD spot detected after expression of the mature form of MCAD in *E. coli* (data not shown). This indicates that the mutant protein is modified post-translationally. In contrast to the case of wild-type MCAD, however, the spot representing nonmodified K304E MCAD is more pronounced. In the black and white reproduction, this is more obvious in the picture from the membrane developed using the more sensitive chemiluminescence substrate (Fig. 2).

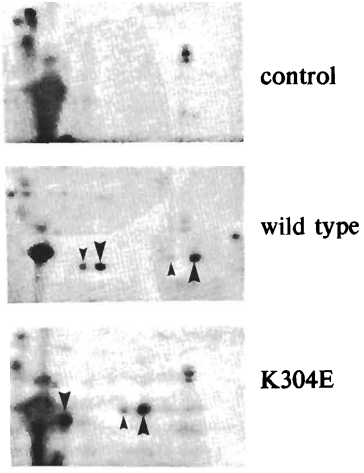

FIG. 3. Two-dimensional gel analysis of wild-type and K304E mutant MCAD in COS-7 cells labeled with [^{35}S]methionine. Cells transfected with plasmids encoding wild-type or K304E pre-MCAD or vector-transfected cells (control) were labeled with [^{35}S]methionine as described under Materials and Methods and analyzed by two-dimensional gel electrophoresis. Sections of the autoradiograms are shown. Upward large and small arrowheads indicate the positions of the nonmodified and modified MCAD spots respectively and downward arrowheads mark spots comprising presumable degradation products (see text).

Pulse Labeling of COS-7 Cells Overexpressing Wild-Type and K304E Mutant MCAD

In order to compare the rate of synthesis of wild-type and K304E MCAD and to study the time order of the modification process, pulse labeling of COS-7 cells was performed with [^{35}S]methionine. Pulse-labeled cells were subjected to 2D gel electrophoresis and autoradiographed. As shown in Fig. 3, four MCAD-specific spots can be detected for wild-type MCAD. Two spots coincide with those observed in silver staining or Western blotting. There are two more spots which are shifted to lower p*I* and slightly lower apparent molecular mass. These are probably degradation products with short truncations of the amino or carboxy terminus or both, as they were not observed in steady state, and therefore might be due to the high expression rate leading to overloading and degradation of part of the newly synthesized MCAD. No spot for pre-MCAD was observed under the conditions used.

For K304E MCAD two spots coinciding with those found in steady state are detected. There is also one spot for a possible degradation product and a second one might be masked by the strong COS-7 cell-specific spot. The intensity of the K304E-specific spots is similar to those observed for wild-type MCAD. This demonstrates that the rate of production of proteolytically processed wild-type and K304E MCAD is similar.

For both wild-type and K304E MCAD, the spot representing the nonmodified form is predominant. When compared to the picture from steady-state analysis of wild-type protein, where the spot representing the modified form is more pronounced, this reveals that the modification step occurs after removal of the leader peptide and indicates that the modification step is rate limiting.

2D Patterns of Primary Human Fibroblasts and Lymphoblastoid Cells from MCAD-Deficient Patients

The material usually available from patients with MCAD deficiency is primary human fibroblasts. It would therefore be desirable to be able to analyze the amounts and the ratio of modification of the respective MCAD polypeptides present in these cells. We investigated the 2D patterns of wild-type and mutant MCAD in primary human fibroblasts from patients by immunoblotting using anti-MCAD antibodies. Figure 4 shows immunoblotting of a 2D gel of normal primary human fibroblasts. Two discrete spots for wild-type MCAD can be detected. The spot representing the modified form is distinctly

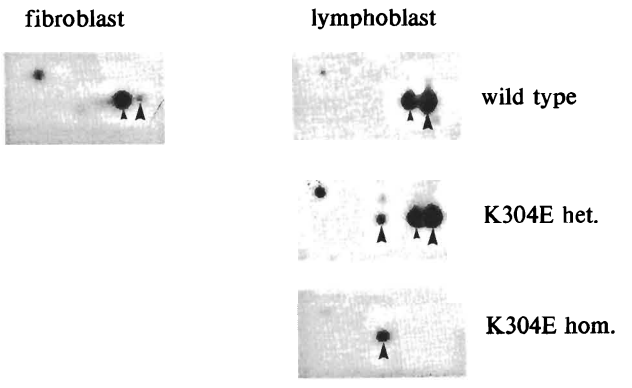

FIG. 4. Two-dimensional gel electrophoresis of primary human fibroblasts and lymphoblastoid cells. Sections from the films after immunostaining of the separated MCAD polypeptides (with chemiluminescence detection; for details see Materials and Methods) from normal primary human fibroblasts, normal lymphoblastoid cells, lymphoblastoid cells heterozygous (K304E het.), and lymphoblastoid cells homozygous (K304E hom.) for the K304E mutation are shown. Positions of the respective nonmodified (large arrowheads) and modified (small arrowheads) MCAD polypeptides are indicated by arrowheads. The spot in the upper left corner of the section is due to cross-reactivity of the antibody with an unknown cellular protein.

more pronounced indicating that this is the predominant form in steady state. All efforts to detect mutant MCAD in primary human fibroblasts from patients failed.

We also investigated lymphoblastoid cell lines from a patient homozygous for the K304E mutation and heterozygous carriers of the mutation. As displayed in Fig. 4, wild-type MCAD can readily be detected. The relative amount of MCAD per cell is higher than in primary human fibroblasts, as the signal for MCAD was clearly more intense. The spots for both the modified and the nonmodified wild-type MCAD protein are observed. In this case the nonmodified spot is somewhat more pronounced. This suggests that lymphoblastoid cells display either a higher rate of protein synthesis or a lower rate of modification at steady state than primary fibroblasts.

A spot representing nonmodified K304E MCAD can be detected both in lymphoblastoid cells homozygous for the mutation and in lymphoblastoid cells heterozygous for K304E. No spot representing modified K304E MCAD could be detected. This may suggest that the rate of modification is impaired or that the modification renders the mutant protein much more unstable. As highlighted in the heterozygous sample, the spot for K304E mutant MCAD is distinctly weaker than the spots for wild-type MCAD. This is in accordance with the data from overexpression in COS-7 cells and the findings by us and others that the K304E mutant protein is distinctly less stable (15,16). The picture here shows directly the relative amounts of wild-type and mutant MCAD in the same cells.

DISCUSSION

The two-dimensional gel electrophoresis system used in this study has a broad range immobilized pH gradient in the first and a 10% homogenous SDS–PAGE in the second dimension, and it allows resolution of MCAD variants with different p*I* caused by exchange of single-charged residues.

The system was set up using heterologously expressed wild-type MCAD and variants carrying point mutations. From comparison of prokaryotic and eukaryotic expression, we demonstrate that MCAD is modified after transport into the mitochondria and proteolytic processing. The modification causes a shift in the isoelectric point to more acidic pH. However, the p*I* shift is smaller than that observed for an exchange of a charged residue or that expected if the amino terminal amino group would be blocked. It must therefore be due to introduction of an acidic function with a p*K* value in the range of the isoelectric point or removal of the positive charge in one or several histidine residues.

In related work with MCAD purified from pig kidney, NMR and chemical analysis indicated that the protein contains one phosphate residue per monomer in addition to the two phosphates of FAD (Büttner, Macheroux, and Ghisla, in preparation). Furthermore, the failure of N-terminal amino acid sequencing indicated, that the protein is N-terminally blocked (Bender, Ghisla, and Rasched, unpublished data). No N-terminal blocking, however, has been observed for MCAD purified from rat (28) and the recombinant human enzyme expressed in *E. coli* (Rasched, Bross, and Ghisla, unpublished data). Therefore, a blocked amino terminus is not compatible with the results found here for human MCAD.

Given the knowledge about covalent post-translational modifications of proteins (29), the simplest explanation compatible with our present data would be phosphorylation of an acidic residue. This would result in loss of the negatively charged carboxyl function and gain of two acidic functions from the phosphate, one of which would be partially ionized at a pH around 7. In sum this would add approximately 0.5 negative charges to the net charge at the isoelectric point of the wild-type protein and could explain the observed shift in p*I*.

Analysis of cells expressing K304E MCAD supported the notion that the mutant is distinctly more unstable than the wild-type protein (15,16). This was furthermore confirmed by comparing the steady-state amounts with the rate of production in pulse labeling. The rate of synthesis and proteolytic processing of K304E MCAD is similar to the wild-type protein, but the steady-state amount is dramatically decreased in relation to the wild-type.

By immunoblotting, K304E MCAD could be detected in lymphoblastoid cells but not in primary human fibroblasts. For wild-type MCAD, the partition between the modified and nonmodified forms is different in primary human fibroblasts and lymphoblastoid cells. In the former, the modified spot is clearly the predominant form, while in the latter the intensity of both spots is similar with the nonmodified one being somewhat stronger. This suggests that the extent of the modification varies between the cell types and may indicate a cell-type specific and regulatory role of the modification.

For K304E mutant MCAD, only the nonmodified spot can be observed in lymphoblastoid cells. It has been shown previously that K304E MCAD protein is impaired in folding and oligomer assembly leading to proteolytic degradation of accumulating intermediates. The present result indicates that the mutation has an effect on the folded and assembled polypeptide also. Two possible explanations for the predominance of the nonmodified form may be envisioned: (i) the modified form of the mutant protein is extremely unstable; and (ii) the mutant protein may not be modified with the same efficiency as the wild-type. A precise molecular characterization of the modification and its biological implications will be necessary in order to be able to decide between these alternatives.

An application of the 2D gel electrophoresis system described will be the analysis of compound heterozygosity in order to study the possible interaction of different MCAD variants with one another. It is possible that mutants affecting tetramer assembly might have a positive or negative effect on the assembly process if they are present in the same cell and thereby lead to stabilization or destabilization of the protein. Lymphoblastoid cells from heterozygous patients or double-transfected COS-7 cells encoding two different MCAD variants (including wild-type) can be used as a model system to study the stability of both proteins when they are produced in the same cells.

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