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Research Article

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Biosynthesis of Electron Transfer Flavoprotein in a Cell-free System and in Cultured Human Fibroblasts

Defect in the Alpha Subunit Synthesis Is a Primary Lesion in Glutaric Aciduria Type II

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Abstract

We investigated the biosynthesis of electron transfer flavoprotein (ETF) in a cell-free system. Both α- (α-ETF, 32,000 molecular weight [mol wt]) and β-subunits (β-ETF, 27,000 mol wt) were nuclear-coded, and synthesized in the cytosol. α-ETF was synthesized as a precursor (pα-ETF), 3,000 mol wt larger than its mature counterpart, and was translocated into the mitochondria and processed to the mature α-ETF. The newly synthesized β-ETF was the same as the mature β-ETF. Using [^{35}S]methionine labeling, we also studied the biosynthesis in cultured normal human fibroblasts. pα-ETF was detected when the cells were labeled in the presence of dinitrophenol or rhodamine 6G. Among six glutaric aciduria type II (GAII) and two ethylmalonic-adipic aciduria cell lines, defective pα-ETF synthesis was observed in three GAII cell lines, and β-ETF synthesis was normal. In one of them, no pα-ETF was synthesized at all, while in another, a faint pα-ETF band of normal size was detected, and was efficiently processed. In the third line, α-ETF was 1,000 mol wt smaller than the normal counterpart, both as the precursor and as the mature form.

Introduction

Electron transfer flavoprotein (ETF)[1] is a soluble mitochondrial flavoprotein (1). It is a dimer of nonidentical subunits (2–6). ETF serves as a specific electron acceptor for several dehydrogenases, including five acyl-CoA dehydrogenases (1, 4, 5), glutaryl-CoA dehydrogenase (7), and sarcosine dehydrogenase (8). ETF transfers electrons from the primary dehydrogenases to the main mitochondrial respiratory chain via ETF/ubiquinone oxidoreductase (ETF-dehydrogenase), an iron-sulfur flavoprotein (9). A genetic deficiency of ETF or ETF-dehydrogenase was considered to be the cause of glutaric aciduria type II (GAII) (10, 11) and its milder variant, ethylmalonic-adipic aciduria (EMA) (12). These metabolic disorders are characterized by severe acidosis, hypoglycemia, and, often, neonatal death. Large amounts of aliphatic mono- and dicarboxylic acids accumulate in blood and urine of patients with these diseases (13). In fact, an ETF-dehydrogenase deficiency has recently been shown with the immunoblotting technique and assay of its activity, to be the cause of GAII in some patients (14). Data on remaining GAII patients suggested an ETF deficiency (14, 15). However, the precise nature of the molecular basis of ETF deficiency remained ambiguous.

In the past several years, it has been shown that many mitochondrial matrix enzymes are nuclear-coded and synthesized on cytoplasmic polyribosomes as precursor peptides 2,000–10,000 molecular weight (mol wt) larger than the respective mature counterpart. The precursors are then translocated into the mitochondria and processed by an energy-dependent process and their extended sequence clipped (16–18). The mechanisms of in vitro translation and posttranslational processing of the precursors by isolated mitochondria have been extensively studied for a number of enzymes (16–18), but those for ETF subunits have not been studied.

In this paper, we report in vitro translation of ETF in a cell-free system using rabbit reticulocyte lysate programmed by rat liver mRNA, and its posttranslational processing by isolated rat liver mitochondria. We also studied the biogenesis of ETF in normal cultured human fibroblasts and those from GAII and EMA patients.

Methods

Materials. L-[^{35}S]Methionine (<600 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL) Inactivated *Staphylococcus aureus* cells, rabbit reticulocyte translation kit, and ^{14}C-labeled standard proteins were obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Sources of GAII and EMA cell lines. The patients from whom the following cell lines were derived have previously been studied: lines 493 (12), 605 (19), 1313 (20); and 1341 (21). Cell line 741 was provided by Dr. F. Walther, St. Annadal Hospital, Maastricht, the Netherlands, and lines 1312, 1391, and 1411 were gifts of Dr. W. J. Rhead, University of Iowa, Iowa City, IA. Lines 605, 1312, 1313, 1341, 1391, and 1411 were from patients with typical severe GAII. Lines 493 and 741 were from patients with EMA (12).

Antibody against ETF. ETF was purified to homogeneity from rat liver mitochondria (5, 22). Antiserum against the pure rat ETF was raised in a rabbit and partially purified as previously described (22). The antiserum specifically precipitated both α- (α-ETF) and β-subunits (β-ETF) of ETF from rat and human liver homogenates. 100 μl of the antiserum was capable of immunoprecipitating ~20 μg of pure ETF.

Cell-free translation and posttranslational processing. The biosynthesis of proteins was carried out using [^{35}S]methionine and a rabbit reticulocyte lysate translation kit according to the method suggested by the supplier, with some modifications. The translation kit was supplemented with 0.6

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1. *Abbreviations used in this paper:* DNP, dinitrophenol; EMA, ethylmalonic-adipic aciduria; ETF, electron transfer flavoprotein; α-ETF, α-subunit of ETF; β-ETF, β-subunit of ETF; GAII, glutaric aciduria type II; NETS, NaCl/EDTA/Triton X-100/SDS buffer; pα-ETF, precursor α-ETF; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; v1, 2, and 3, variant alleles for α-ETF.

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A260 U of calf liver tRNA and 3 μg of rat liver poly(A^+) RNA per 100 μl of translation mixture. Rat liver RNA was prepared according to the method of Conboy et al. (23). Poly (A^+) RNA was isolated using an oligo(dT) column (type 2; Collaborative Research, Lexington, MA) according to the supplier's procedure.

Posttranslational processing experiments using the isolated rat liver mitochondria were essentially carried out according to the method of Fenton et al. (24). Mitochondria were prepared according to the method of Lowenstein et al. (25). Freshly prepared mitochondria were suspended in a buffer consisting of 2 mM Hepes, pH 7.4, 220 mM mannitol, 70 mM sucrose, supplemented with 2 mM EGTA, 4 mM MgCl, 4 mM ADP, and 20 mM glutamate. The final concentration of mitochondrial suspension was 4 mg protein/ml. The translation mixture (30 μl) was mixed with an equal volume of the mitochondrial suspension, and incubated at 30°C for the period indicated. After incubation, the reaction mixture was fractionated to supernatant and mitochondrial pellet. Products of the cell-free translations or processing reactions were solubilized in 9 vol of a buffer containing 150 mM NaCl, 10 mM EDTA, pH 7.4, 0.5% Triton X-100, 0.25% sodium dodecyl sulfate (SDS), and 2% unlabeled methionine (NETS/methionine-buffer), and immunoprecipitated as described below.

Labeling of fibroblasts in culture. The confluent monolayer of fibroblasts in 6-cm dishes (~5×10^6 cells/dish) was labeled with [^{35}S]methionine (50–100 μCi) in 5 ml of labeling media containing 60% Puck's saline F, 15% dialyzed fetal calf serum, and 10% glucose, as previously described (26). The dishes were incubated at 37°C for 1 h. After removal of the labeling medium, cells were washed with phosphate-buffered saline and harvested by adding 1 ml of NETS/methionine buffer to each dish. The solubilized cell extract was subjected to immunoprecipitation. When rhodamine 6G or dinitrophenyl (DNP) was used, they were added to the medium at a final concentration of 2.1 μM (rhodamine 6G) or 4 mM (DNP) 30 min before the medium was replaced with the labeling medium containing [^{35}S]methionine. For pulse-labeling experiments, the cells were labeled in the same manner as described above for 1 h in the presence of 4 mM DNP. After the cells were washed twice with phosphate-buffered saline, the medium was replaced with the growing medium with or without DNP.

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis (PAGE). 1 ml of sample preparation solubilized by NETS/methionine-buffer was centrifuged for 30 min at 105,000 *g*. The supernatant was mixed with 10 μl of anti-ETF antibody. The immune complexes were recovered by adding 10 vol of *S. aureus* cell suspension per volume of antibody and subsequently washed as described (26). Slab SDS-PAGE was performed using 10% gels (0.8 mm thick) according to the method of Laemmli (27). Gels were stained with Coomassie Brilliant Blue, treated with Autofluor (National Diagnostics, Inc., Somerville, NJ), dried, and fluorographed according to the supplier's directions.

Results

Cell-free translation of ETF. When cell-free translation was carried out using [^{35}S]methionine, rabbit reticulocyte lysate, and rat liver poly(A^+) RNA, and its products were analyzed by immunoprecipitation and SDS-PAGE, two sharp radiolabeled protein bands were detected (Fig. 1, lane *2*). One of them was identical in size (27,000 mol wt) to β-ETF. In contrast, the molecular weight of the other band (35,000 mol wt) was ~3,000 mol wt larger than the α-ETF (lane *1*), indicating that α-ETF was synthesized as a larger precursor (pα-ETF). Intensity of these labeled bands was very weak and only barely detectable when an excess amount of the pure rat ETF was added to the translation mixture, before the addition of the antiserum, to compete for the antibody with the labeled proteins (lane *3*). Also, no labeled band was detected in a control (lane *4*) in which anti–rat ETF antiserum was replaced with a nonimmune rabbit serum.

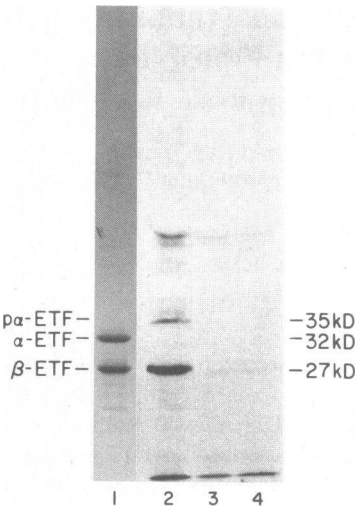

Figure 1. Slab SDS-PAGE of ETF synthesized in a cell-free system. Unlabeled pure ETF was also analyzed for comparison. Cell-free translation was carried out using [^{35}S]methionine in a rabbit reticulocyte lysate system programmed by rat liver poly(A^+)RNA. *Lanes 2–4* are fluorograms. *Lane 1,* pure rat ETF stained by Coomassie Blue; *lane 2,* cell-free translation mixture immunoprecipitated with anti–rat ETF antiserum; *lane 3,* cell-free translation mixture immunoprecipitated after an addition of 10 μg of unlabeled pure rat ETF; and *lane 4,* cell-free translation mixture treated with nonimmune rabbit serum. kD, mol wt.

The intensity of radiolabel of the pα-ETF band was always weaker than that of the β-ETF in repeated experiments.

Import and processing of ETF subunits by isolated rat liver mitochondria. The products of a cell-free translation mixture were incubated with intact isolated rat liver mitochondria. The mixture was then separated into supernatant and mitochondrial pellet, and the two fractions were separately immunoprecipitated and electrophoresed. As shown in Fig. 2, pα-ETF and β-ETF were detected in the supernatant. The amounts of both bands significantly decreased with time, but the decrease of pα-ETF was more drastic. In the mitochondrial pellet, the processed α-ETF was detected along with pα-ETF and β-ETF at 10 min. The amounts of α- and β-ETF increased thereafter with time, whereas that of pα-ETF remained constant. Pα-ETF is presumably bound to the mitochondrial membrane, as observed in the processing of acyl-CoA dehydrogenase precursors (28). The molecular sizes of the processed α- and β-subunits were essentially identical to those of the purified rat ETF.

Biosynthesis of ETF in cultured human fibroblasts. Cultured normal human fibroblasts were incubated with [^{35}S]methionine, and the labeled products were analyzed by immunoprecipitation with anti–rat ETF antiserum and SDS-PAGE. Two sharp labeled bands were observed as shown in Fig. 3, lane *1*. Molecular sizes (32,000 and 27,000 mol wt) of these bands were identical to those of rat α- and β-ETF. These bands were not detected in the competition experiment shown in lane *2,* in which an excess of the purified rat ETF was added before the addition of the antiserum, or in the control in which antiserum was replaced with nonimmune rabbit serum (lane *3*), confirming the identity of these labeled bands as α- and β-ETF.

Demonstration of pα-subunit by the use of DNP and rhodamine 6G, and kinetics of precursor processing in cultured human fibroblasts. It has previously been shown that processing of precursors of mitochondrial matrix enzymes were inhibited by the inhibitors of mitochondrial energy metabolism such as DNP and rhodamine 6G (24, 28, 29). When normal human fibroblasts were incubated with [^{35}S]methionine in the presence of rhodamine 6G (Fig. 4) or DNP (Fig. 5 *A*), labeled pα-ETF (35,000 mol wt) and β-ETF (27,000 mol wt) were detected. No

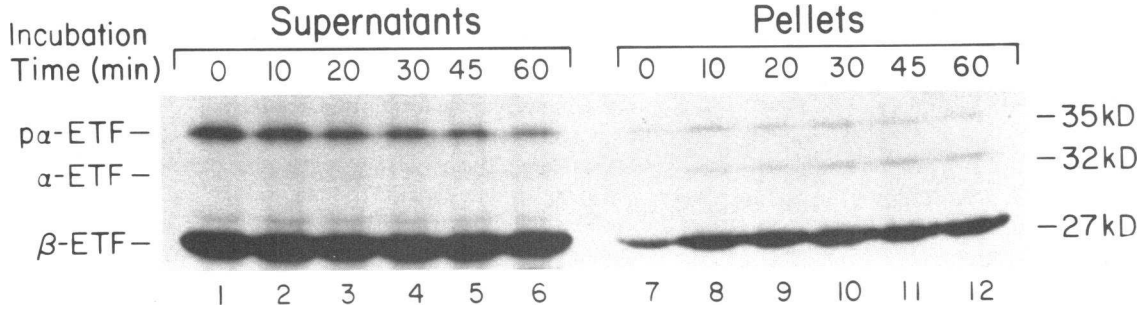

Figure 2. Time course of import and processing of ETF subunits by isolated rat liver mitochondria. [^{35}S]Methionine-labeled products in the translation mixtures (30 μl each) were incubated at 30°C with an equal volume of an isolated rat liver mitochondria suspension (4 mg protein/ml) for the period indicated. After incubation, the samples were centrifuged at 8,000 *g* for 5 min to separate the supernatants and mitochondrial pellets. Both preparations were immunoprecipitated with anti-rat ETF antiserum and electrophoresed. Only the relevant portion of the gel fluorogram is shown. kD, mol wt.

α-ETF was detected. The syntheses of pα-ETF and β-ETF were both strongly inhibited by 500 μg/ml of cycloheximide (Fig. 4, lanes *5, 6, 9* and *10*) but was not inhibited at all by 100 μg/ml of chloramphenicol (Fig. 4, lanes *3, 4, 7* and *8*). The processing of the precursor was not inhibited by the latter as well.

The time course of the processing of precursor ETF to the mature form was studied by pulse-labeling the cells with [^{35}S]methionine in the presence of 4 mM DNP, followed by a chase after removing DNP (Fig. 5 *A*). A small amount of processed α-ETF was detected at 5 min (lane *3*). The half-life of pα-ETF in the conversion to the mature form was ~15 min (lane *5*) as judged by the parity of these two bands.

To study the stability of pα-ETF and β-ETF in the cytosols, the cells were pulse-labeled with [^{35}S]methionine and chased in the presence of DNP (Fig. 5 *B*). The half-life of pα-ETF was slightly <1 h. The half-life of β-ETF, under the same conditions, was between 2 and 3 h. In contrast to the relative instability of pα-ETF and β-ETF in the cytosol, processed α- and β-ETF were considerably more stable in the mitochondria, as determined in the absence of DNP (Fig. 5 *B*, lanes *1* and *2*).

Identification of a defect in the synthesis of the α-subunit and its heterogeneity in fibroblasts from patients with glutaric aciduria type II. The ETF synthesis in two normal, six GAII, and two EMA cell lines were studied using the same method. First, ETF synthesis in the absence of inhibitors was tested (Fig. 6). The intensity and molecular size of α- and β-ETF synthesized by three GAII cell lines (1312, 1341, and 1411) and two EMA cell lines (493 and 741) were indistinguishable from those synthesized by two normal cell lines. In contrast, marked abnormalities in the synthesis of α-ETF were found in the other three GAII cell lines. Cell line 605 did not synthesize α-ETF at all. In 1313, only a faint radiolabeled α-ETF was detected. In 1391, an α-ETF band, as intense as that of the normal α-ETF, was detected, but its moblity on SDS-PAGE was distinctly faster than that of normal α-ETF. In all three lines, the synthesis of the β-subunit was well labeled and its size was normal.

In order to examine whether the defective α-ETF synthesis in the three GAII lines was due to a defect in the synthesis of the pα-ETF, or, alternatively, due to pα-ETF being synthesized but not processed properly, these cells were labeled with [^{35}S]methionine in the presence of rhodamine 6G (Fig. 7). In one normal and two EMA (493 and 741) cell lines, a labeled pα-ETF of 35,000 mol wt was detected. In contrast, no labeled pα-ETF was detected at all in line 605, whereas a weak but distinct radioactive pα-ETF band with normal size was detected in line 1313. In the latter, there was an additional faint band that is slightly smaller than pα-ETF. This band may represent a break-down product of pα-ETF. In this cell line, the intensity of pα-ETF labelled in the presence of rhodamine 6G was stronger

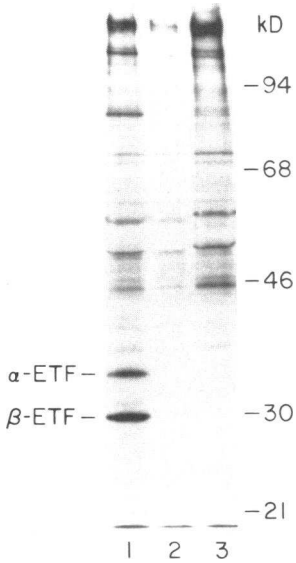

Figure 3. Slab SDS-PAGE of the [^{35}S]methionine-labeled ETF synthesized in cultured normal human fibroblasts. The confluent monolayers of cells were labeled and the cell extracts (1 ml each) were immunoprecipitated by anti-rat ETF antiserum (10 μl each), and the immunoprecipitates were analyzed on 10% SDS-PAGE as described in Methods. *Lane 1,* labeled α- and β-subunits of ETF immunoprecipitated with anti-rat ETF antiserum; *lane 2,* immunoprecipitates from a cell extract, to which 7 μg of unlabeled pure rat ETF was added before the addition of the antiserum; *lane 3,* a control in which anti-ETF antiserum was replaced with nonimmune rabbit serum. kD, mol wt.

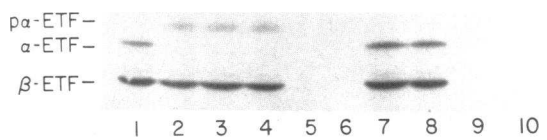

Figure 4. The effects of cycloheximide (CX) and chloramphenicol (CP) on the synthesis of electron transfer flavoprotein subunits in cultured normal human fibroblasts. The basic conditions for labeling, immunoprecipitation, and electrophoresis were the same as Fig. 3. Inhibitors added are as follows: *lane 1,* none; *lane 2,* 2.1 μM rhodamine 6G (Rh); *lane 3,* 2.1 μM Rh and 50 μg/ml CP; *lane 4,* 2.1 μM Rh and 100 μg/ml CP; *lane 5,* 2.1 μM Rh and 0.5 mg/ml CX; *lane 6,* 2.1 μM Rh and 1 mg/ml CX; *lane 7,* 50 μg/ml CP; *lane 8,* 100 μg/ml CP; *lane 9,* 0.5 mg/ml CX; and *lane 10,* 1 mg/ml CX.

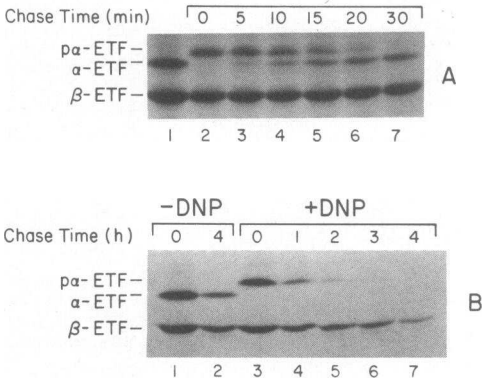

Figure 5. Time course of processing (*A*) and stability of precursor α- and β-subunits of ETF in normal cultured human fibroblasts. Only the relevant portion of the gel fluorogram is shown. (*A*) The confluent cell monolayers were pulse-labeled for 1 h with [^{35}S]methionine in the presence of DNP, and then chased after removing DNP as described. *Lane 1,* mature sized α- and β-subunits labeled in the absence of DNP; *lanes 2–7,* subunits labeled in the presence of DNP and chased after DNP removal. (*B*) The confluent monolayer cells were pulsed for 1 h with [^{35}S]methionine in the absence (*lanes 1* and *2*) and presence of 4 mM DNP (*lanes 3–7*) and chased in the absence (*lanes 1* and *2*) and presence (*lanes 3–7*) of 4 mM DNP. Other experimental conditions are the same as in Fig. 3.

than that of α-ETF labeled in the absence of the inhibitor. In line 1391, a clear band of radioactive pα-ETF was detected, but its size was ~1,000 mol wt smaller than normal pα-ETF.

Discussion

We have shown here, using the cell-free translation system and isolated rat liver mitochondria, that rat α-ETF was synthesized in the cytosol as a precursor (35,000 mol wt) 3,000 mol wt larger than the mature counterpart (32,000 mol wt). The pα-ETF was imported into the mitochondria and processed to the mature form with its extended sequence cleaved, as in the case of many enzymes associated with the mitochondrial matrix or inner membrane (16–18). β-ETF was also synthesized in the cytosol and imported into the mitochondria, but it was synthesized in a form that is indistinguishable from the mature β-subunit in

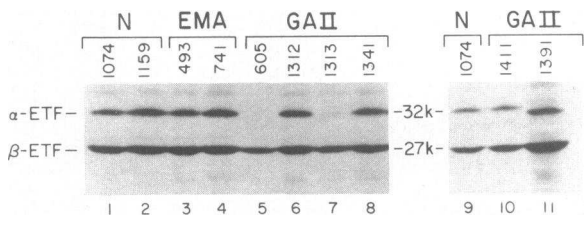

Figure 6. Electrophoretic demonstration of a defect in the synthesis of α-subunit of ETF in three GAII fibroblast lines. Experimental conditions for labeling, immunoprecipitation, and electrophoresis are the same as in Fig. 3. Only the relevant portion of the gel fluorogram is shown. *Lanes 1, 2,* and *9,* normal fibroblast lines (N); *lanes 3–4,* EMA cell lines; *lanes 5–8, 10* and *11* each represents a separate glutaric aciduria type II cell line (GAII). Our cell line numbers are presented at the top of each lane.

size. There were no detectable changes in its molecular size during or after import. Currently, only a few mitochondrial matrix or inner membrane enzymes (proteins) in mammals are known to be synthesized in a form with the same molecular size as that of the mature enzyme. These include mitochondrial 3-oxoacyl-CoA thiolase (30) and adenine nucleotide translocator (31).

Taking advantage of the fact that the anti–rat ETF antibody cross-reacts with human ETF, we studied the ETF biosynthesis in cultured human skin fibroblasts. First, we have shown that the sizes of α- (32,000 mol wt) and β-subunits (27,000 mol wt) of human ETF were identical to the rat counterparts. As in the case of other matrix enzymes (24, 28, 29), posttranslational processing of the pα-ETF was inhibited by DNP and rhodamine 6G. Like rat α-ETF, human α-ETF was synthesized as a precursor (35,000 mol wt), 3,000 mol wt larger than the mature form, while the mass of the newly synthesized β-ETF was the same as that of the mature form. The synthesis of both pα-ETF and β-ETF was severely inhibited by cycloheximide (500 μg/ml) but not at all by chloramphenicol (100 μg/ml). These results confirm that both α- and β-ETF are nuclear-coded and synthesized in the cytosol although the molecular size of the latter does not change after the mitochondrial import. The half-life of pα-ETF (15 min) in the conversion to the mature form is considerably longer than that of pre–aspartate aminotransferase (30–60 s) (32), pre–ornithine carbamyltransferase (up to 2 min) (33), and short- (<5 min), medium- (5 min), and long-chain acyl-CoA dehydrogenases (5 min). It is similar to that of isovaleryl-CoA dehydrogenase (28). pα-ETF and β-ETF were relatively stable in the cytosol. β-ETF was significantly more stable inside the mitochondria than in the cytosol.

We studied the biosynthesis of ETF in cells from six GAII patients and two EMA patients. We have shown for the first time that an abnormal α-ETF synthesis was the primary lesion causing metabolic derangements in three GAII lines. Our results indicate that there are at least three variant alleles for α-ETF. Variant 1 (v1) is incapable of producing translatable mRNA for α-ETF. Line 605 is a homozygote for this allele. v1 may be due to a deletion, nonsense mutation, or a frameshift in a region close to the 5′ end of the genome. Alternatively, v1 allele produces an extremely labile messenger RNA. Variant 2 (v2) allele produces a variant pα-ETF with a normal size, as seen in line 1313. v2 precursor is processed to the variant α-ETF with a normal size in a normal fashion, suggesting that v2 is due to a point mutation in the main sequence. v2 α-ETF in line 1313 was always only faintly labeled. In contrast, its precursor was consistently more intensely labeled than the mature form, suggesting that the mature v2 α-ETF was labile. The intensity of v2 pα-ETF was still considerably weaker than normal pα-ETF. This observation and the presence of a faint extra band that is slightly smaller than pα-ETF both suggest that v2 pα-ETF is also labile. Also, it is likely that line 1313 is a compound heterozygote for v1 and v2 alleles. Alternatively, the immunoreactivity of v2 with anti–rat ETF antibody was weaker than that of normal human α-ETF. Variant 3 (v3) produces a variant α-ETF, the mobility of which on SDS-PAGE is slightly faster than normal α-ETF, suggesting that this variant α-ETF is 1,000 mol wt smaller than the normal counterpart, as seen in line 1391. Since its precursor has a leader sequence of normal size and it was readily processed to the mature form in a normal manner, it appears that this variant is due to a mutation in the region close to the 3′ end of genome, causing premature termination of translation. Alter-

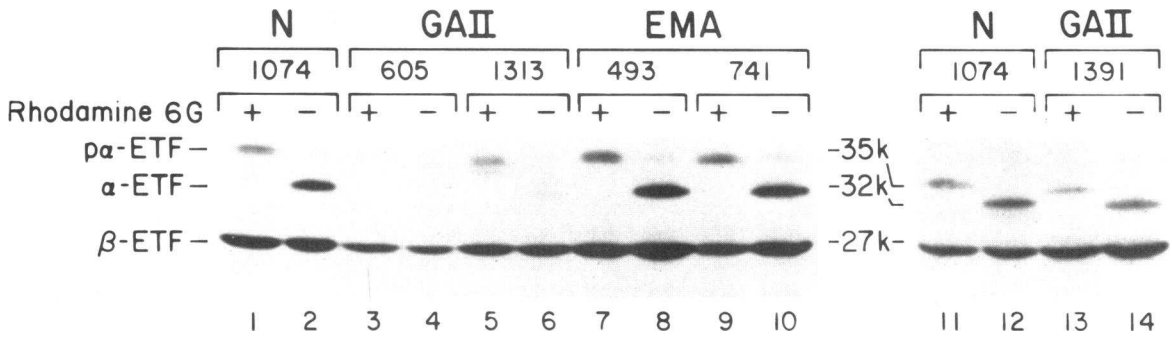

Figure 7. Electrophoretic demonstration of a defect in the synthesis of the α-subunit precursor of ETF in three GAII fibroblasts. Cells were labeled with [^{35}S]methionine in the presence and absence of 2.1 μM rhodamine 6G. Other experimental conditions were the same as in Fig. 3. An equal volume of each extract was analyzed. *Lanes 1, 2, 11,* and *12,* normal fibroblasts (N); *lanes 3–6, 13,* and *14,* each represent a separate glutaric aciduria type II fibroblast (GAII); *lanes 7–10,* ethylmalonic-adipic aciduria cell lines (EMA).

natively, it could be due to a point mutation, resulting in an amino acid substitution that causes changes in the tertiary structure and electrophoretic mobility.

Our present finding on these three GAII cell lines is consistent with the observation by Rhead and associates that ETF activities in these three lines were severely deficient, while in the other GAII or EMA lines deficiencies were milder (15, 34). This paper thus constitutes the first report describing a defect in α-ETF synthesis as the primary lesion that causes GAII in these three patients. Recently, Frerman and Goodman (14) analyzed preexisting unlabeled ETF in several GAII cell lines using an immunoblotting technique. They detected, in lines 605 (their 1196) and 1391 (their 1441), a weak band that had an electrophoretic mobility slightly greater than that of normal human α-ETF. They detected β-ETF in a decreased amount in line 1391, but not at all in line 605. They considered a possibility that the polypeptide with the mobility similar to that of human α-subunit was derived from rabbit serum. They concluded that the significance of the polypeptide in the fibroblast extracts, which cross-reacted with anti-ETF-IgG in their study, was not clear. The reason for their failure to detect β-ETF in line 605 may be that β-ETF is unstable when it does not form a dimer with a normal α-ETF.

Our identification of defects in the pα-ETF synthesis in these three lines, using [^{35}S]methionine labeling and immunoprecipitation as presented in this paper, and the failure of Frerman and Goodman (14), using immunoblotting, to do the same, well illustrates the difference between the information gained by these two methods. Immunoblotting detects immunoreactive proteins that are present in the cells at the time of determination. Therefore, if the pertinent variant protein is unstable, it may quickly decompose as it is synthesized. The pertinent variant protein may not accumulate to a detectable amount. Although the parent protein may be undetectable, immunoreactive degradation products may be detected, hence producing confusing results. In contrast, the [^{35}S]methionine labeling/immunoprecipitation method detects immunoreactive proteins that are being synthesized by the cells with an extremely high sensitivity. It is capable of unambiguously identifying genetic defects that result either in the synthesis of a variant protein grossly differing from its normal counterparts in molecular features, or in a gross decrease or complete lack of synthesis.

We have shown in the present study that in the three other GAII and two EMA lines, the ETF synthesis was normal. During the preparation of this paper, Frerman and Goodman have reported that these five cell lines were deficient in the activity of ETF-dehydrogenase (14, 35). Thus, it is now clear that GAII and its milder variant, EMA, are due to a defect of either α-ETF or ETF-dehydrogenase. Theoretically, a defect of β-ETF can also cause GAII or EMA, but no such mutants have yet been identified.

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