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Deficiency of electron transfer flavoprotein or electron transfer flavoprotein:ubiquinone oxidoreductase in glutaric acidemia type II fibroblasts

(inherited disease/acyl-CoA dehydrogenases/electroblotting/crossreacting material)

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ABSTRACT **Glutaric acidemia type II (GA II) is a human genetic disorder. It has been suggested that the primary defect in this disorder is a deficiency of a protein involved in electron transport between the acyl-CoA dehydrogenases and the bc_1 complex of the mitochondrial respiratory chain. Antisera were raised to purified porcine electron transfer flavoprotein (ETF) and electron transfer flavoprotein:ubiquinone oxidoreductase (ETF:QO). The antisera were used to detect the two electron transferases in control and GA II fibroblasts by immunoblotting. Fibroblasts from three unrelated GA II patients were deficient in immunologically detectable ETF:QO and extracts from these three fibroblast lines contained no detectable ETF:QO catalytic activity. Fibroblasts from parents of two of these patients had ETF:QO activity intermediate between activities in control fibroblasts and fibroblasts from the patients. These data indicate that the primary defect in these patients is a deficiency of ETF:QO and that the mode of transmission of the gene is autosomal recessive. Fibroblasts from two other patients with severe GA II had normal levels of ETF-QO activity and antigen but were deficient in immunoreactive ETF. These findings show that GA II results from a deficiency of ETF in some patients and ETF:QO in others. In addition, these investigations provide strong evidence for the specificity and physiological function of the iron–sulfur flavoprotein ETF:QO.**

Glutaric acidemia type II (GA II) is an inherited metabolic disease characterized by nonketotic hypoglycemia and the accumulation and excretion of large amounts of several organic acids (1–10). Most of these acids are clearly derived from the acyl-CoA substrates of six mitochondrial flavoprotein dehydrogenases (ref. 11; unpublished data)‡ that transfer electrons to the mitochondrial respiratory chain via electron transfer flavoprotein (ETF) (12, 13) and ETF:ubiquinone oxidoreductase (ETF:QO), an iron–sulfur flavoprotein (14). Some patients also excrete sarcosine (2, 4), the substrate of another ETF-linked flavoprotein dehydrogenase (15). The oxidation of substrate precursors of ETF-linked acyl-CoA dehydrogenases by intact GA II fibroblasts is greatly impaired (1, 3, 9); however, *in vitro* assays of those primary flavoprotein dehydrogenases show normal catalytic activities (2–4, 7). Whole cell oxidation by GA II fibroblasts of succinate and pyruvate is also normal, indicating that electron flux from NADH and succinic dehydrogenases through the bc_1 region of the respiratory chain to oxygen is not defective (1, 3, 9). These observations have been interpreted as suggesting that electron transfer from the primary flavoprotein dehydrogenases to the bc_1 complex of the respiratory chain is defective in GA II patients (3, 4). We have reported previously a >75% decrease of the characteristic electron paramagnetic resonance signal ($g_z = 2.08$) of the reduced iron–sulfur cluster of ETF:QO in liver submitochondrial particles from one GA II patient. These particles also lacked material that crossreacted with antibody to the porcine ETF:QO (16).

The work reported here extends our investigations to cultured skin fibroblasts from the patient described previously (16), five additional GA II patients, and parents of two GA II patients. These studies demonstrate that GA II can be caused by a deficiency of ETF or ETF:QO. The data indicate that the genes for defective ETF:QO and α subunit of ETF are transmitted in an autosomal recessive fashion and provide strong evidence for the specificity of the iron–sulfur flavoprotein.

METHODS

Cell Culture. GA II fibroblast lines 1196 (1), 1730 (6), 1441 (7), 1716 (10), KH (2), and 1691 (16) were from unrelated patients who have been described previously. Fibroblasts were grown in Eagle's minimal essential medium containing Earle's salts, 10% fetal bovine serum, nonessential amino acids, and antibiotics.

Enzyme Preparations and Assays. Human fibroblast acyl-CoA dehydrogenases in the soluble fractions of sonically disrupted cells were assayed fluorometrically as described by Frerman *et al.* (17). The sedimented membrane fractions from these preparations were suspended in 10 mM Tris·HCl (pH 7.4) containing 0.5% Triton X-100 and used as the source of human fibroblast ETF:QO in enzymatic assays and in immunoblotting experiments (see below). Human liver mitochondria were prepared by the procedure of Lemaster and Hackenbrock (18) from material obtained by autopsy. The liver mitochondria were suspended in 10 mM Hepes buffer (pH 7.8) containing 0.1 mM EDTA, 5% ethylene glycol, 2 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$), 1 mM tosylamide-2-phenylethyl chloromethyl ketone (TPCK), and 1 mM tosyl-L-lysine chloromethyl ketone (TLCK) and disrupted by sonication for 60 sec at 4°C. The preparations were centrifuged at 100,000 × *g* for 1 hr at 4°C to obtain soluble and particulate fractions, which were used as sources of human ETF and ETF:QO, respectively. Mitochondrial membrane fractions (7 mg of protein per ml), suspended in 10 mM Tris buffer (pH 7.4) containing 250 mM sucrose and 1 mM dithiothreitol, were extracted with 2.5% Triton X-100 for

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Abbreviations: GA II, glutaric acidemia type II; ETF, electron transfer flavoprotein; ETF:QO, ETF:ubiquinone oxidoreductase; Q, ubiquinone; $PhMeSO_2F_1$ phenylmethylsulfonyl fluoride; TPCK, tosylamide-2-phenylethyl chloromethyl ketone; TLCK, tosyl-L-lysine chloromethyl ketone; mu, milliunits.

‡Human and pig liver glutaryl-CoA dehydrogenases reduce ETF to the anionic semiquinone.

30 min at 4°C. The supernatant fraction obtained after centrifugation at 100,000 × g for 1 hr was used as the source of crude human ETF:QO for catalytic assays.

ETF:QO activity was assayed fluorometrically under anaerobic conditions by following the decrease in fluorescence of oxidized ETF flavin in the ETF:QO-catalyzed comproportionation of ETF hydroquinone (ETF_{hq}) and oxidized ETF (ETF_{ox}) that yields ETF semiquinone (ETF_{sq}) (19): $ETF_{ox} + ETF_{hq} \leftrightarrows 2\ ETF_{sq}$. This reaction will be discussed in detail elsewhere (unpublished data). Membrane fractions of fibroblasts were extracted with 0.5% Triton X-100 in 10 mM Tris·HCl (pH 7.4) and centrifuged at 100,000 × g. The pellet was reextracted and centrifuged as before and the two supernatant fractions were combined. The two extractions solubilize 92–96% of ETF:QO activity. ETF hydroquinone was prepared by careful titration of the protein with $Na_2S_2O_4$ under anaerobic conditions. Assay mixtures (about 0.75 ml) containing 2-amino-1,3-propanediol hydrochloride (pH 8.6) and glucose were prepared in a fluorescence cuvette sealed with a 1-cm-thick rubber stopper and made anaerobic by evacuation and purging with argon; 3 units of glucose oxidase and 20 units of catalase were added. After equilibration at 25°C for 10 min, ETF hydroquinone and anaerobic oxidized ETF were added with gas-tight syringes. The final reaction mixtures, 0.8 ml, contained 20 mM 2-amino-1,3-propanediol hydrochloride, 20 mM glucose, glucose oxidase and catalase, 1.5 μM ETF hydroquinone, and 0.3 μM oxidized ETF. The reactions were initiated by addition of the crude enzyme preparations (1–20 μg of fibroblast protein). Initial velocities were determined by analyses of progress curves of fluorescence decay (20, 21). Reactions were carried out at 25°C with excitation at 342 nm; emission was measured at 496 nm (21). One milliunit of activity is equivalent to 1 nmol of ETF_{ox} reduced per min at 25°C.

Catalytic activity of ETF in the soluble fraction of human liver mitochondria was assayed spectrophotometrically as described by Hauge (22) and modified by Rhead and Amendt (23).

Protein was estimated by the procedure of Lowry *et al.* (24) as modified by Miller (25) using bovine serum albumin as the standard.

Preparation of Antibodies. Antisera were raised in New Zealand White rabbits (about 3 kg) to porcine ETF and ETF:QO as described by Inui *et al.* (26) except that the initial immunizations were with 250 μg of each protein; subsequent immunizations were with 25 μg. Thirty micrograms of the anti-ETF:QO IgG preparation completely inhibited 18 milliunits (mu) of human ETF:QO activity and 80 μg of the anti-ETF IgG preparation inhibited 9 mu of human ETF activity to a maximum of 90%. Preimmune sera did not inhibit either activity.

Electrophoresis and Protein Blotting. Soluble proteins from human fibroblasts for immunoblotting were obtained after sonication of fibroblasts in 10 mM Tris·HCl (pH 7.4) containing 2 mM $PhMeSO_2F$, 1 mM TPCK, and 1 mM TLCK. The disrupted cells were centrifuged for 1 hr at 100,000 × g and the resulting supernatant fractions were electrophoresed and blotted. Proteins from soluble and particulate fractions of fibroblasts were subjected to polyacrylamide gel electrophoresis (PAGE) on 10% gels in the presence of $NaDodSO_4$ as described by Laemmli (27). Proteins were transferred electrophoretically to nitrocellulose (28) and were detected with the appropriate IgG preparation and ^{125}I-labeled staphylococcal protein A as described (29).

Materials. Acyl-CoA substrates were purchased from P. L. Biochemicals. Pig liver ETF was prepared by the method of Husain and Steenkamp (30). ETF preparations had an A_{270}:A_{436} ratio of 5.6 and were homogeneous, as judged by $NaDodSO_4$/PAGE. The concentration of ETF was determined spectrophotometrically, ϵ_{436} = 13.4 mM^{-1} (13). ETF:QO was purified from pig liver submitochondrial particles as described by Ruzicka and Beinert (14) and modified by Beckmann and Frerman (31). The preparation gave a single band on $NaDodSO_4$/PAGE and isoelectric focusing in 6% polyacrylamide gels in the presence of 0.05% Triton X-100. General acyl-CoA dehydrogenase was prepared as described (32). Radioiodinated protein A (4.88 mCi/mg; 1 Ci = 37 GBq) was the generous gift of Kent Wilcox (Department of Microbiology, Medical College of Wisconsin) and was prepared with the Bolton–Hunter reagent supplied by New England Nuclear (2000 Ci/mmol).

RESULTS

Table 1 shows that the specific activities of acyl-CoA dehydrogenases in five GA II fibroblast lines are not significantly different from those in control fibroblasts. Glutaryl-CoA dehydrogenase activity is also normal in the fibroblast line 1441 (7) and liver mitochondria of the patient from whom the line 1691 was derived (unpublished data). Glutaryl-CoA dehydrogenase activity has been shown to be normal in line KH (2). These data are consistent with previous reports that individual acyl-CoA dehydrogenase activities are not deficient in GA II (2–4, 7), which has been described as a multiple acyl-CoA dehydrogenase deficiency (7, 9, 10, 16).

Fig. 1 shows proteins in the particulate fractions from control and GA II whole fibroblast sonicates that crossreact with anti-porcine ETF:QO. Control fibroblast membranes (Fig. 1 *A* and *B*, lanes 3–5) contain crossreacting material with the same electrophoretic mobility as the oxidoreductase purified from porcine liver (M_r = 69,000) (Fig. 1 *A* and *B*, lane 1) and a protein present in human liver submitochondrial particles (Fig. 1 *A* and *B*, lane 2). GA II lines 1196 and 1441 (Fig. 1*A*, lanes 6 and 7) also contain this polypeptide, but, in GA II lines 1691, 1716, and 1730 (Fig. 1*B*, lanes 6, 7, and 8, respectively), the polypeptide is absent. In line 1691, small amounts of the polypeptide became evident when the blot was overexposed, consistent with our previous observation that the EPR signal of the reductase was reduced, but not absent, in liver submitochondrial particles of the patient (16); lines 1716 and 1730 did not show crossreacting material even when the blots were overexposed.

Table 2 shows the specific activity of ETF:QO in extracts of control and GA II fibroblasts. The three lines deficient in ETF:QO antigen contain no detectable ETF:QO activity. In addition, no ETF:QO activity was detected in fibroblast line KH from the GA II patient reported by Gregersen *et al.* (2). Extracts of fibroblasts from both parents of two of those patients, 1691 and 1730, contain reduced levels of ETF:QO activity, indicating that they are heterozygous for the

Table 1. Acyl-CoA dehydrogenase activities in control and GA II human fibroblast lines

Fibroblasts	Specific activity, mu/mg of protein			
	C_4-CoA	C_8-CoA	C_{16}-CoA	Iv-CoA
Control				
Range	1.00–2.08	1.85–3.66	2.49–5.41	1.48–4.08
Mean ± SD*	1.57 ± 0.40	2.71 ± 0.72	4.09 ± 1.06	2.69 ± 1.09
GA II				
1196	1.12	2.25	2.51	2.83
1441	1.27	2.14	2.76	1.85
1691	0.85	2.39	3.30	1.74
1716	0.74	1.89	1.34	1.86
1730	ND	3.30	ND	ND

C_4-CoA, butyryl-CoA; C_8-CoA, octanoyl-CoA; C_{16}-CoA, palmitoyl-CoA; and Iv-CoA, isovaleryl-CoA. ND, not determined.
*n = 8.

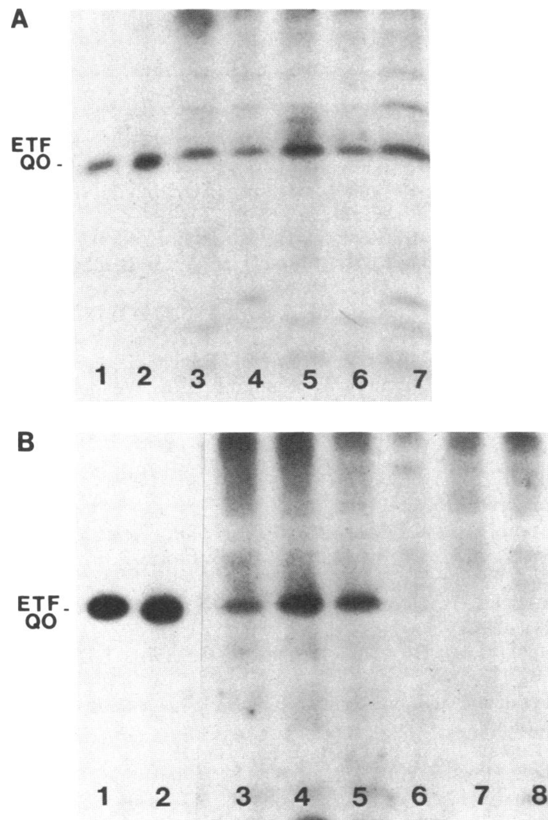

FIG. 1. Immunoblots of ETF:QO in control and GA II fibroblast membranes. Fibroblast membrane proteins, 70 μg, were separated on 10% polyacrylamide gels, transferred electrophoretically to nitrocellulose, and detected by antibody overlay and ^{125}I-labeled protein A. (*A*) Lane 1, purified porcine liver oxidoreductase, 80 ng; lane 2, human liver submitochondrial particles, 10 μg; lanes 3–5, control fibroblast membranes; lane 6, GA II line 1196; lane 7, GA II line 1441. (*B*) Lane 1, purified porcine liver oxidoreductase; lane 2, human liver mitochondrial membranes; lanes 3–5, control fibroblasts; lane 6, GA II line 1691; lane 7, GA II line 1716; lane 8, GA II line 1730.

ETF:QO defect. These data strongly support the hypothesis that the deficiency of ETF:QO is the primary defect in these GA II patients and that the deficiency is transmitted as an autosomal recessive trait. Fibroblast lines 1196 and 1441, from two other severely affected GA II patients, contain normal levels of ETF:QO antigen and catalytic activity.

The immunoblot in Fig. 2 shows an analysis of control and GA II fibroblast extracts for ETF subunits. The mass of the α subunit of human ETF, designated α_H (lane 2), is 300–500 daltons less than the mass of the porcine α subunit (M_r = 31,000) (13); the mass of the human β subunit is apparently the same as the porcine β subunit (M_r = 27,000) (13). Both ETF subunits were detected in normal fibroblasts (lanes 3–5) and the ETF:QO-deficient lines 1691, 1716, 1730, and KH (lanes 8–11). In contrast, GA II lines 1196 and 1441, which contain ETF:QO antigen and catalytic activity, are deficient in ETF subunits. In line 1196 (lane 6) neither the α nor the β subunit was detected; however, after a longer exposure (shown in the *Inset* of Fig. 2), a polypeptide with a greater mobility than the normal α subunit was detected. Line 1441 has slightly reduced levels of both ETF subunits, as judged by the intensity of the ^{125}I signal (lane 7). The α subunit in these GA II fibroblasts is also abnormal, as judged by its greater electrophoretic mobility relative to the normal human α subunit. The significance of the polypeptide in the fibroblast extracts that crossreact with anti-ETF IgG is not clear. It was not detected in extracts of human mitochondria (lane 2). A polypeptide with similar electrophoretic mobility was observed with preimmune serum.

Table 2. ETF:QO activity in control and GA II human fibroblast lines

Fibroblasts	Specific activity, mu/mg of protein
Control	
Range	10.9–20.9
Mean ± SD*	15.4 ± 3.6
GA II	
1196	13.5
1441	12.7
1691	≤0.10
1716	≤0.10
1730	≤0.10
KH	≤0.10
Parents†	
1755	6.2
1756	8.4
1785	4.9
1786	3.6

*n = 10.
†Lines 1755 and 1756 are from parents of patient 1691 and lines 1785 and 1786 are from parents of patient 1716.

DISCUSSION

Based on immunological evidence and direct enzymatic assay, GA II is the result of the deficiency of ETF:QO in some patients and ETF in others. The immunological data define deficiencies of specific subunits of ETF and are consistent with the report of Rhead and Amendt (23), who showed that line 1441 contains 30% residual ETF activity. More recently, they have shown that lines 1196 and 1441 contain 5% and 8%, respectively, of normal ETF activity (W. Rhead, personal communication). Their preliminary data indicate that line KH, which has no detectable ETF:QO activity and normal levels of ETF subunits, has 40% of control ETF activity.

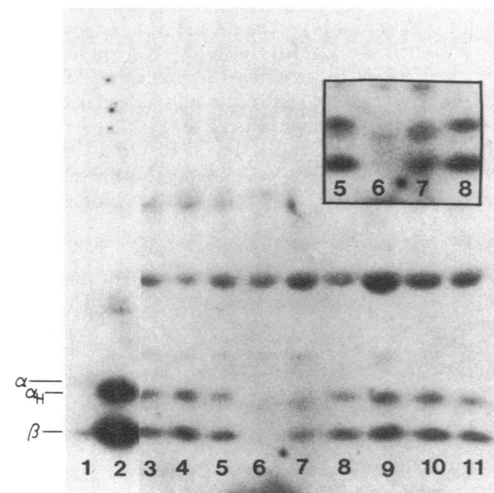

FIG. 2. Immunoblots of ETF subunits in extracts of control and GA II fibroblasts. Pig liver ETF, 60 ng (lane 1), human liver soluble mitochondrial protein, 28 μg (lane 2), and human fibroblast soluble proteins, 50 μg (lanes 3–11), were separated on 10% polyacrylamide gels, transferred to nitocellulose, and detected by antibody overlay and ^{125}I-labeled protein A. The extracts are from control fibroblasts (lanes 3–5) and the GA II lines 1196 (lane 6), 1441 (lane 7), 1691 (lane 8), 1716 (lane 9), 1730 (lane 10), and KH (lane 11). (*Inset*) Lanes 5–8 after a longer exposure. The symbol α_H refers to the human α subunit.

It has been suggested that neonatal GA II is transmitted as an X-linked trait in some families (6). Our data indicate that ETF:QO deficiency is transmitted as an autosomal recessive trait. The data also suggest that the gene for the α subunit of ETF is not X-linked, because line 1441 was derived from a female with an apparently affected sister (7). Further, Rhead *et al.* (33) have shown that fibroblasts from the parents of this child oxidize [1-^{14}C]butyrate and [2-^{14}C]leucine at diminished rates. X-linkage of the gene coding for the β subunit of ETF cannot be ruled out at this time.

It has been noted that some GA II patients have congenital anomalies, notably polycystic kidneys (16). We have no information about patient 1730; however, the other patients with ETF:QO deficiency had congenital anomalies (refs. 10 and 16; N. Gregersen and E. Christensen, personal communication). The patients with ETF deficiency did not have congenital anomalies (1, 7). It is not clear at this time whether the presence of congenital anomalies correlates better with the protein affected by the mutation or the severity of the deficiency, since no patient without detectable ETF activity has been reported.

The role of the iron–sulfur flavoprotein ETF:QO in fatty acid β oxidation was initially proposed by Ruzicka and Beinert (14), who showed that the iron–sulfur cluster of the protein was rapidly reduced by butyryl-CoA in the presence of the general acyl-CoA dehydrogenase and ETF. The reduction of the iron–sulfur cluster was dependent on ETF. Further, the iron–sulfur flavoprotein catalyzed electron transfer from ETF to coenzyme Q_1. Additional evidence for the function of the iron–sulfur flavoprotein is based on the observation of a 5- to 10-fold increase in the EPR signal of the oxidoreductase, relative to the signal of center 2 of the NADH dehydrogenase complex, in brown adipose tissue during cold-acclimation of guinea pigs (34). Fatty acid β oxidation is a principal oxidative pathway supporting thermogenesis in this tissue. The results presented in this paper constitute strong additional evidence for the specificity of the oxidoreductase, since its deficiency is accompanied by the accumulation and excretion of substrates, or metabolites of substrates, derived from the substrates of virtually all known ETF-linked flavoprotein dehydrogenases.

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