Am. J. Hum. Genet. 40:257–266, 1987

Deficient Fumarase Activity in an Infant with Fumaricacidemia and Its Distribution between the Different Forms of the Enzyme Seen on Isoelectric Focusing

R. PETROVA-BENEDICT,* B. H. ROBINSON,* T. E. STACEY,† J. MISTRY,† AND R. A. CHALMERS†

*Department of Pediatrics and Biochemistry, University of Toronto, and the Research Institute, The Hospital for Sick Children, Toronto; and †Division of Inherited Metabolic Diseases, Clinical Research Centre, Harrow, Middlesex, United Kingdom

SUMMARY

A male infant, whose parents were first cousins, presented at 6 mo of age with hypotonia, microcephaly, and delayed development. He was found to have large amounts of fumaric and succinic acids present in the urine. In lysed cultured skin–fibroblast preparations, the activity of fumarase was found to be 22.7% of that in controls. Cell fractionation by homogenization and by digitonin treatment indicated that the residual activity in the cells of the patient was primarily located in the mitochondrial fraction rather than in the cytosolic fraction. Isoelectric focusing of fibroblast extracts showed that six bands of fumarase activity were discernible in control cell lines, two of them cytosolic with pI's of 5.53 and 5.60 and four of them mitochondrial with a pI of 5.65–6.8. In contrast, isoelectric focusing of fibroblast extracts from the fumarase-deficient patient showed only a single band of activity with a pI corresponding to the mitochondrial type seen in the controls. Immunoprecipitation of proteins with rabbit antifumarase antibody in (^{35}S)-methionine-labeled fibroblasts indicated that a protein of correct size (M_r = 44,000 daltons) corresponding to fumarase was synthesized in similar amounts in both the patients and controls. It is proposed that in the patient's cells a single active species of fumarase that is mitochondrial in location is synthesized. Since it is known that

Received August 22, 1986.

Address for correspondence and reprints: Dr. Brian H. Robinson, Department of Pediatrics and Biochemistry, University of Toronto, and the Research Institute, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada.

mitochondrial and cytosolic fumarases are encoded by the same gene but differ slightly in amino acid sequence, it is possible that a point mutation might explain these findings.

INTRODUCTION

Fumarate hydratase (E.C. 4.2.1.2) occurs in two subcellular compartments, the cytosol and the mitochondria in isoenzymic forms (Hill and Teipel 1972; Tolley and Craig 1975). In most humans tissue analysis has shown that there are six electrophoretically different forms of fumarase, three of them cytosolic and three of them mitochondrial in location (Edwards and Hopkinson 1979*b*). In rare individuals as many as 14 electrophoretic species can be identified, and in these cases the activity of fumarase is reduced (Edwards and Hopkinson 1979*a*, 1979*b*). The existence of this genetic variant, because both cytosolic and mitochondrial isoenzymes were affected by it, gave rise to the suggestion that both isoenzymes were determined by the same autosomal gene locus (Edwards and Hopkinson 1979*a*).

Recently two lines of evidence have lent support to this suggestion. O'Hare and Doonan (1985) have shown, in tryptic digests of mitochondrial and cytosolic fumarase from pig liver, that 58 tryptic peptides are held in common, with two peptides unique to the mitochondrial isoenzyme and five peptides unique to the cytosolic form. The mitochondrial isoenzyme N-terminal amino acid is alanine while the cytosolic isoenzyme N-terminal amino acid is glutamate or glutamine (O'Hare and Doonan 1985). Furthermore, it was found that the amino acid composition was almost identical for the two isoenzymes and that it was difficult to differentiate between them on immunological grounds (Kobayashi et al. 1981). Though both isoenzymes are thought to be tetrameric (M_r = 180,000 daltons), the mature subunits having a relative molecular weight of 45,000 daltons, it has been shown that rat liver mRNA translation in vitro produces two distinct fumarase proteins, one at M_r = 45,000 and one at M_r = 50,000 (Ono et al. 1985). The larger species is incorporated into rat liver mitochondria in the process undergoing proteolytic cleavage to 45,000 daltons. Thus, rat liver mRNA contains two separate messages encoding fumarase, one destined for the mitochondria and one presumably destined for the cytosol (Ono et al. 1985).

A patient with fumaricacidemia, mental retardation, and speech impairment but no apparent deficiency in enzyme activity has recently been reported (Whelan et al. 1983). In this paper we describe a second case of fumaricaciduria in a mentally retarded child in which fumarase is deficient both in the mitochondrial and cytosolic compartments.

CASE REPORT

F.K. was born after a normal pregnancy to parents who were first cousins, the mother having had a bout of influenza at 6 mo that did not appear to have a

disturbing effect on the pregnancy. Apart from some feeding difficulties, no abnormality was noticed until the sixth month, when the child was noticed to have a small head size (below the first percentile), hypotonia, and slow development. The child was investigated at the Queen Elizabeth Hospital for Sick Children and found to have increased levels of fumaric acid (360 mg/g creatinine) and succinic acid (160 mg/g creatinine) in his urine on a chronic basis. (These findings will be described in detail in a separate report [Chalmers et al., in press].) There is an older male sibling who is normal and a maternal uncle who is mentally retarded. A skin fibroblast culture from this child was established and investigated in Harrow and in Toronto.

MATERIAL AND METHODS

Cell Culture

Human skin fibroblasts were grown from explants and cultured in Eagles minimal essential medium (α-MEM) with 15% added fetal calf serum. Cultures were either from the patient with fumaricaciduria or from age-matched controls.

Preparation of Mitochondrial and Cytosolic Fractions

Two types of mitochondrial and cytosolic preparations were used to determine the activity of fumarase in different cell fractions. In the first technique the cells were scraped from the culture dish and disrupted by homogenization (glass on glass) in a medium containing 0.25 M sucrose, 5 mM Tris HCl, pH 7.4, 1 mM ethylenediaminetetraacetate (EDTA). The homogenate was centrifuged at 600 *g* for 10 min to remove cell debris, and the resulting supernatant was centrifuged at 14,000 *g* for 10 min to produce a mitochondrial fraction (pellet) and a cytosolic fraction (supernatant).

In the second technique the cells on the culture dish were drained of tissue culture medium, washed with phosphate-buffered saline (PBS), and covered with an ice-cold solution containing 0.25 M sucrose, 20 mM morpholino propane sulphonate (MOPS), 2 mM EDTA, 0.8 mg digitonin/ml, pH 7.4. After 3 min the supernatant was removed and replaced by one containing 0.25 M sucrose, 20 mM MOPS, 1 mM EDTA sucrose MOPS Etta (SME), pH 7.4. After 5 min this solution was removed and the extracted cells were scraped into 0.2 ml SME. The digitonin supernatant was used to determine the cytosolic enzyme activity, and the scraped pellet was used to determine the mitochondrial activity.

(^{35}S)-Methionine-labeling Experiments

Radioactive labeling of cells was performed using the method described by Lam Hon Wah et al. (1983), with some modifications. Fibroblasts were grown to early confluence in 75-cm^2 tissue-culture flasks, one per cell strain per experiment. One day prior to radioactive labeling, the medium was removed from the flasks and replaced by 10 ml of α-MEM modified to contain 10 μM methionine. The next day, the medium was replaced by 5 ml of the same medium supple-

mented with 200 μCi of (^{35}S)-methionine/ml (800–1,200 Ci/mmol; Amersham, Arlington Heights, IL); the cells were allowed to incubate overnight; and the radioactive medium was removed. After two washes with HEPES-buffered saline, to each flask was added 1.5 ml of SME buffer containing 0.8 mg digitonin/ml. After 5 min the supernatant was removed and kept on ice as the "cytosolic" fraction. To each flask was then added 1.5 ml of a buffer containing 0.15 M NaCl, 30 mM HEPES, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% Triton X-100. This buffer solubilized the remaining cellular constituents except the cytoskeletons (Osborn and Weber 1977). After 1 h the supernatant was decanted and kept as the "mitochondrial" fraction. To the cytosolic and mitochondrial extracts an equal volume of CCl_4 was added, and the resulting solution was vortexed. This solution was then centrifuged at 15,000 *g* for 10 min; the supernatant was adjusted to 2% Triton X-100 and 0.4% sodium dodecyl sulfate (SDS), and 10 μl of normal rabbit serum was added. After 1 h 100 μl (packed volume) of PBS-washed Pansorbin (formalin-fixed *Staphylococcus aureus* containing protein A; Calbiochem, San Diego, CA) was added and the mixture was incubated at room temperature (4 C) for 30 min (Vesterberg 1972). After centrifugation at 15,000 *g* for 10 min, the supernatants were taken, 15 μl of either normal rabbit serum or antifumarase antiserum was added, and after 2 h incubation at room temperature, 15 μl of Pansorbin was added. The tubes were incubated for a further 1 h and centrifuged at 15,000 *g* for 10 min, and the pellets were washed four times with a medium containing 0.15 M NaCl, 10 mM Tris HCl, pH 7.4, 5 mM EDTA, 1 mM PMSF, 0.1% Triton X-100, and 1 μg methionine/ml. The resultant pellets were resuspended by boiling for 5 min in 4 μl of 0.03 M Tris HCl, pH 6.8, 1% SDS 10% glycerol, and 0.2 M dithiothreitol for running on 7.5% polyacrylamide slab gels. (^{35}S)-labeled protein bands were visualized using fluorography. Exposure of the X-ray plates was for 2 days.

Isoelectric Focusing

Isoelectric focusing was performed via the method of Vesterberg (1972), using 120 × 160-mm thin-layer gels prepared with 5% acrylamide and 1.8% ampholines, pH 3.5–10, and 0.13% ampholines, pH 4–6 and 5–7. The LKB multiphor system was used, the cathodic reservoir being 1 M NaOH and the anodic reservoir being 1 M H_3PO_4. Electrophoresis was carried out at 20 W for 3 h at 3 C. Fumarase activity was detected on the gels via the staining procedure described by Edwards and Hopkinson (1979*b*) after washing the gels in PBS.

Measurement of Fumarase Activity

Fumarase activity was measured by monitoring the dehydration of L-malate spectrophotometrically at 250 mM at 30 C in 0.05 sodium phosphate buffer, pH 7.6 (Freiden et al. 1954) containing 0.05% Triton X-100.

Preparation of Antibodies to Pig Heart Fumarases

Antifumarase antiserum was prepared by intramuscular injection of 0.5 mg dialyzed pig heart fumarase (Boehringer-Mannheim) into a rabbit, followed 4

wk later by a booster of 0.5 mg injected subcutaneously. Preimmune sera were collected and used as normal rabbit sera. Antifumarase antiserum collected 6 wk after the initial injection was found to possess activity such that 2 μl of antiserum would inhibit the activity of 12 ng fumarase by 80%.

RESULTS

Fumarase activity was measured in whole-cell extracts and in cell fractions prepared via either mechanical disruption or digitonin treatment. In whole cells lysed by the detergent Triton X-100, the activity in the cells of the patient was 22.7% of that observed in control cells. In a post–14,000 *g* supernatant following mechanical disruption of the cells with a glass-on-glass homogenizer, the activity from the patient's cells was 10.6% of that prepared from control cells. The post–14,000 *g* mitochondrial pellet from the patient showed 22% of the activity of the equivalent fraction from control cells. In a cell fractionation achieved using digitonin treatment, cytosolic activity was undetectable in the patient's cells and was 19.1% of the activity in the mitochondrial fraction compared with the control cells. These results suggest that both mitochondrial and cytosolic fumarase activities are deficient in this patient, with the deficiency being more pronounced in the cytosolic fraction (table 1).

Whole-cell extracts from cultured skin fibroblasts were prepared by sonicating the cells in 1% glycine. After a brief period of centrifugation to remove cell debris (20,000 *g* for 10 min), these extracts were applied to an isoelectric-focusing gel (fig. 1). The control cells typically showed four to six major bands on staining for fumarase activity. The cells of the patient, on the other hand, displayed only one band of activity with a pI of ~6.0. Isoelectric focusing of cells fractionated by differential centrifugation gave two predominant bands with pI's of 5.53 and 5.60 for the cytosolic fraction (fig. 2). In contrast, the mitochondrial fraction gave four predominant bands of pI 5.65–6.8. The fractions obtained from the fibroblasts of the fumarase-deficient patient via differ-

TABLE 1

FUMARASE ACTIVITY IN CULTURED SKIN FIBROBLASTS IN PATIENT (P) AND CONTROLS (C)

	MEAN ± SEM (*N*) FUMARASE ACTIVITY IN (nmol/min/mg protein)		P/C (%)
	P	C	
Whole-fibroblast extract[a]	14.9 ± 1.5 (6)	65.7 ± 3.7 (6)	22.7
Cytosol fraction[b]	7.4 ± 1.7 (3)	69.6 ± 9.2 (3)	10.6
Mitochondrial fraction	41.3 ± 4.4 (3)	187.1 ± 3.8 (3)	22.1
Digitonin-soluble fraction (cytosol)[c]	0 (5)	36.3 ± 3.1 (5)	0
Digitonin-insoluble fraction (mitochondria)	18.0 ± 2.6 (6)	94.2 ± 9.2 (6)	19.1

NOTE.—*N* = Number of determinations.

[a] Whole cells disrupted by 0.05% Triton X-100.

[b] Whole cells disrupted by mechanical homogenization and separated into cytosolic and mitochondrial fractions as described in Material and Methods.

[c] Whole cells treated with 0.8 mg digitonin/ml, as described in Material and Methods, to give cytosolic and mitochondrial fractions again.

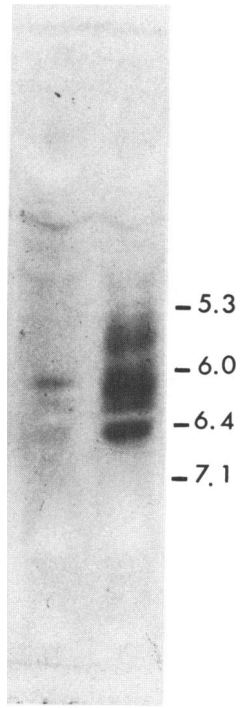

FIG. 1.—Isoelectric focusing gel of whole-cell extracts of patient's and controls' cultured skin fibroblasts stained for fumarase activity. Isoelectric focusing was performed as described in Material and Methods, and the resultant gel was stained for fumarase activity. Cells were harvested from petri dishes by scraping, washing in PBS, and resuspending the packed cells after centrifugation in 1% glycine, pH 7.0. This suspension was sonicated to disrupt the cells, the solution was centrifuged at 20,000 *g* for 10 min, and 2 mg protein of the supernatant was applied to the gel. Lane 1: Fumarase-deficient cell line 1616. Lane 2: Control cell line 1685.

ential centrifugation showed little discernible activity on staining of the gels, only one faint mitochondrial band being visible.

In order to determine whether the lack of fumarase activity was due to either defective activity of protein species synthesized in normal amounts or to defective synthesis of the actual amount of these protein species, experiments were performed to evaluate the incorporation of (^{35}S)-methionine into fumarase. Cultured skin fibroblasts were grown in a culture medium containing (^{35}S)-methionine and then lysed with Triton X-100 or separated by digitonin fractionation into mitochondrial and cytosolic fractions. These extracts were immunoprecipitated with antifumarase antibody raised in rabbits as described in Material and Methods, and the immunoprecipitates were separated using SDS–polyacrylamide gel electrophoresis (SDS-PAGE). When these gels were examined using fluorography, a prominent band (M_r = 44,000 daltons) was produced in cell extracts immunoprecipitated with antifumarase antiserum but was not

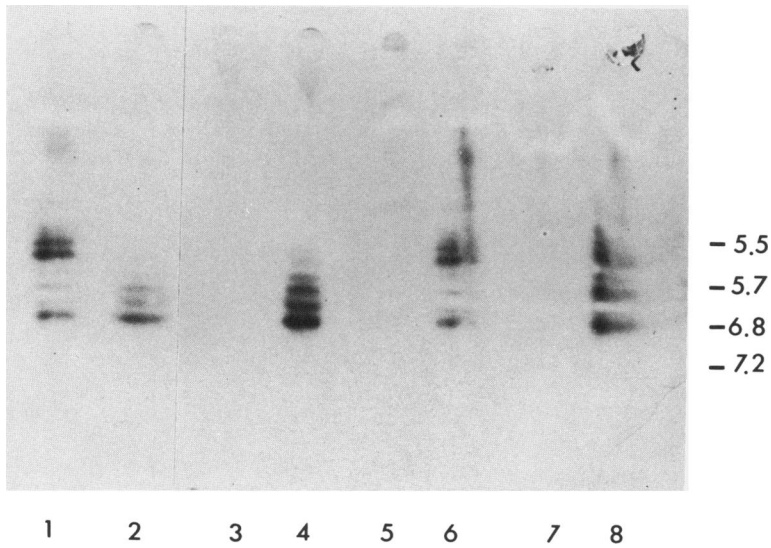

FIG. 2.—Fumarase activity in mitochondrial and cytosolic fractions of cultured skin fibroblasts after isoelectric focusing. Cultured skin fibroblasts (12 mg protein) were disrupted by mechanical homogenization, and mitochondrial and cytosolic fractions were prepared by differential centrifugation. The mitochondrial pellets were dissolved in 100 μl of 1% glycine, pH 7.0, and sonicated. The cytosolic fraction was concentrated by Amicon filtration, and to the final volume (50 μl) was added 20 μl 1% gycine, pH 7.0. Aliquots (40 μl) of these fractions were applied to the gel. Lane 1: Control cell line 1685, cytosolic fraction. Lane 2: Control cell line 1685 mitochondrial fraction. Lane 3: Deficient cell line 1616, mitochondrial fraction. Lane 4: Control cell line 1206, mitochondrial fraction. Lane 5: Deficient cell line 1616, cytosolic fraction. Lane 6: Control cell line 1206, cytosolic fraction. Lane 7: Whole-cell extract cell line 1616. Lane 8: Whole-cell extract cell line 1685.

produced in parallel extractions with preimmune serum (fig. 3). This band ran identically to the band obtained with fumarase run on the same gel and stained with Coomassie blue (results not shown). The intensity of the band obtained using fluorography was the same in the cells from the patient as it was in control cells. When fractions obtained using digitonin treatment were used, it was also found that the amounts of (^{35}S)-labeled fumarase in the cytosolic and mitochondrial fractions were the same in control cells as they were in the corresponding fractions of the patient's cells as shown in a parallel experiment (fig. 4).

DISCUSSION

We have shown that cultured skin fibroblasts from a patient with fumaric-aciduria have a deficiency in the enzyme fumarase, a deficit that is evident in both the cytosolic and the mitochondrial species of this enzyme as judged by activity. The cytosolic enzyme appears to be more severely affected than the mitochondrial, as is borne out by the isoelectric-focusing data, which shows only one band, a mitochondrial one that is active, compared with the two cytosolic and four mitochondrial bands that are seen in controls. Evidence

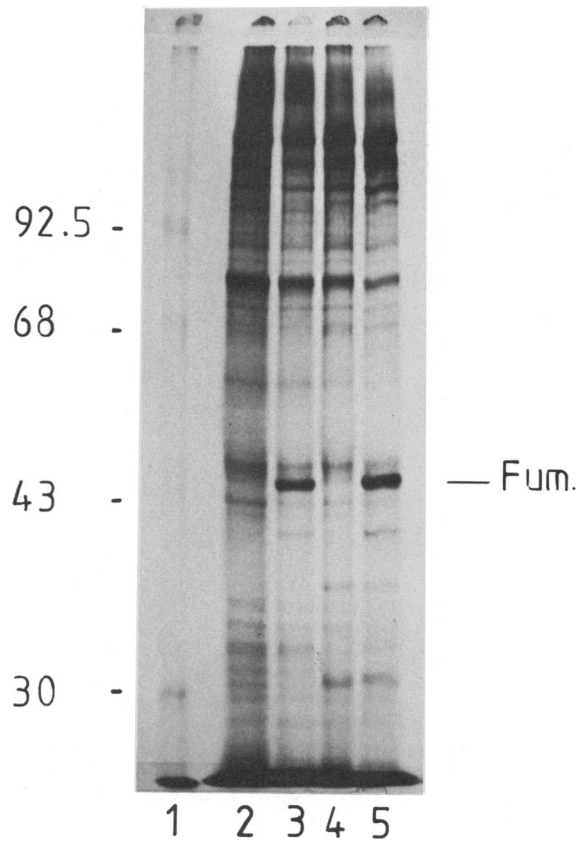

FIG. 3.—SDS-PAGE of proteins, immunoprecipitated by antifumarase antiserum from extracts of cultured skin fibroblasts grown in (^{35}S)-methionine-containing medium. Cells were labeled and extracted as described in Material and Methods. The cell extracts were then immunoprecipitated by either preimmune serum or with antifumarase antiserum. These immunoprecipitates were then run on an SDS/10% polyacrylamide gel, and labeled proteins were visualized by fluorography. Lane 1: Protein standards. Lane 2: Control cell line 1685 immunoprecipitated with preimmune serum. Lane 3: Control cell line 1685 immunoprecipitated with antifumarase antiserum. Lane 4: Deficient cell line 1616 immunoprecipitated with preimmune serum. Lane 5: Deficient cell line 1616 immunoprecipitated with antifumarase antiserum.

from (^{35}S)-methionine incorporation into immunoprecipitable fumarase suggests that, despite the low enzymic activity, fumarase is synthesized in the correct amounts both in the cytosolic and in the mitochondrial compartments.

The above data are highly suggestive of a mutation affecting a single amino acid change. The question posed by the presence of residual activity concentrated in just one of the mitochondrial isoelectric-focusing bands is an intriguing one. Since the N-terminal amino acids and two tryptic peptides of the cytosolic and mitochondrial fumarases are different, there is ample evidence that the primary structure of the two isoenzymes is not identical (O'Hare and Doonan 1985). However, because both isoenzymes are encoded at the same genetic locus, the mature mRNA species for the synthesis of the two isoenzymes must

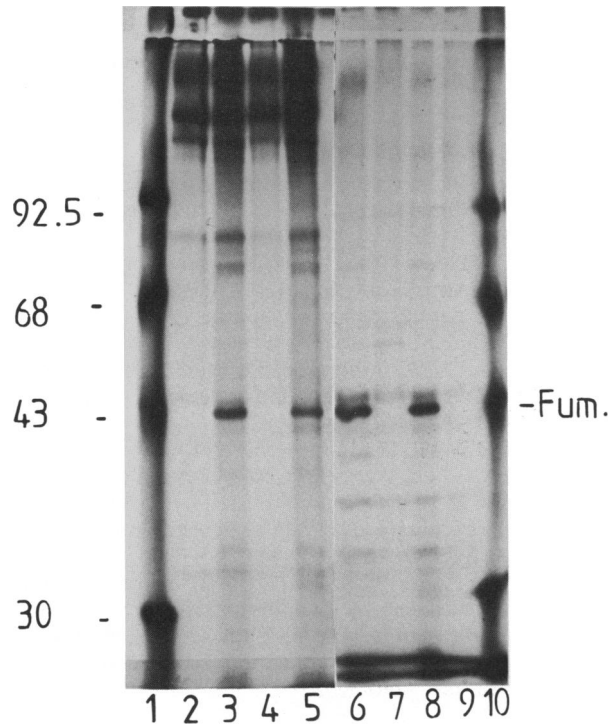

FIG. 4.—SDS-PAGE of proteins immunoprecipitated from cytosolic and mitochondrial fractions by antifumarase antiserum. Experiments were carried out as described in Material and Methods. Cells grown on (^{35}S)-methionine were fractionated by digitonin treatment into mitochondrial and cytosolic fractions. These fractions were then immunoprecipitated by either preimmune serum or antifumarase antiserum. Immunoprecipitates were run on SDS/10% polyacrylamide gels. Separate gels were run for cytosolic and mitochondrial fractions. Lanes 1 and 10: Protein standards. Lanes 2 and 3: Control cell line 1685 cytosol. Lanes 4 and 5: Deficient cell line 1616 cytosol. Lanes 6 and 7: Control cell line 1685, mitochondria. Lanes 8 and 9: Deficient cell line 1616 mitochondria. Lanes 2, 4, 7, and 9 were immunoprecipitated with preimmune serum. Lanes 3, 5, 6, and 8 were immunoprecipitated with antifumarase antibody.

be produced by differential splicing of the same genetic transcript. This situation would allow the presence of unique mutations affecting either mitochondrial or cytosolic activities exclusively. The nature of the chemical differences between the isoelectric-focusing forms of these enzymes is unknown. Since they are not glycoproteins (O'Hare and Doonan 1985), it is unlikely that post-translational modification by glycosylation is responsible. Sequential modification of a single residue in each subunit of the tetrameric form might, however, explain the presence of four distinguishable isoelectric species in the mitochondrial compartment. Despite the mutation, one of these species retains catalytic activity in the mitochondrial enzyme from the patient's cells. A possible alternative hypothesis is that each of the mitochondrial forms arises by differential proteolytic cleavage of the N-terminal end of the molecule, one form remaining active because of either its unique configuration or removal of the amino acid residue affected by the mutation.

ACKNOWLEDGMENTS

We thank the Medical Research Council of Canada and the National Foundation the March of Dimes (U.S.) for financial support.

REFERENCES

Chalmers, R. A., T. Stacey, B. M. Tracey, J. Mistry, and V. G. Oberholzer. Diagnosis and prenatal diagnosis of fumaric aciduria due to fumarase deficiency. J. Inherited Metab. Dis. (in press).

Edwards, Y. H., and D. A. Hopkinson. 1979*a*. Further characterisation of the human fumarase variant FH 2-1. Ann. Hum. Genet. **43**:103–108.

———. 1979*b*. The genetic determination of fumarase isoenzymes in human tissue. Ann. Hum. Genet. **42**:303–308.

Freiden, C., R. M. Bock, and R. A. Alberty. 1954. Studies of the enzyme fumarase. II. Isolation and physical properties of crystalline enzyme. J. Am. Chem. Soc. **76**:2482–2485.

Hill, R. L., and J. W. Teipel. 1972. Fumarase and crotonase. Pp. 539–568 *in* P. Boyer, ed. The enzymes. Vol. **5**. 3d ed. Academic Press, New York and London.

Kobayashi, K., T. Yomanishi, and S. Tuboi. 1981. Physiochemical, catalytic and immunochemical properties of fumarases crystallised separately from mitochondrial and cytosolic fractions of rat liver. J. Biochem. **89**:1923–1931.

Lam Hon Wah, A. M., K. F. Lam, F. Tsui, B. Robinson, M. E. Saunders, and R. A. Gravel. 1983. Assignment of the α and β chains of human propionyl-CoA carboxylase to genetic complementation groups. Am. J. Hum. Genet. **35**:889–900.

O'Hare, M. C., and S. Doonan. 1985. Purification and structural comparisons of the cytosolic and mitochondrial isoenzymes of fumarase from pig liver. Biochim. Biophys. Acta **827**:127–134.

Ono, H., N. Yoshimura, M. Sato, and S. Tuboi. 1985. Translocation of proteins into rat liver mitochondria: existence of two different precursor polypeptides of liver fumarase and import of the precursor into mitochondria. J. Biol. Chem. **260**:3402–3407.

Osborn, M., and K. Weber. 1977. The detergent-resistant cytoskeleton of tissue culture cells includes the nucleus and the microfilament bundles. Exp. Cell Res. **106**:339–349.

Tolley, E., and I. Craig. 1975. Presence of two forms of fumarase (fumarate hydratase E.C. 4.2.1.2) in mammalian cells: immunological characterisation and genetic analysis in somatic cell hybrids: confirmation of the assignment of a gene necessary for the enzyme expression to human chromosome 1. Biochem. Genet. **13**:867–871.

Vesterberg, O. 1972. Isoelectric focussing of proteins in polyacrylamide gels. Biochim. Biophys. Acta **257**:11–19.

Whelan, D. T., R. E. Hill, and S. McClorry. 1983. Fumaric aciduria: a new organic aciduria, associated with mental retardation and speech impairment. Clin. Chim. Acta **132**:301–307.