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Immunochemical studies on cultured fibroblasts from patients with inherited methylmalonic acidemia

(genetic disease/cobalamin-dependent enzyme/radioimmunoassay/crossreacting material)

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ABSTRACT **We developed a radioimmunoassay to quantitate material crossreacting immunochemically with human methylmalonyl-CoA mutase (methylmalonyl-CoA CoA-carbonylmutase, EC 5.4.99.2), and have applied this assay to extracts of fibroblasts from controls and from 32 patients with methylmalonic acidemia due to inherited deficiencies in mutase activity. Four control lines had an average of 237 ng of crossreacting material (CRM) per mg of cell protein (range, 193–297 ng/mg). Mutant lines from each of the four *cbl* complementation groups of inherited methylmalonic acidemia, which have normal amounts of mutase activity *in vitro*, contained quantities of CRM comparable to those of control lines. On the other hand, 28 cell lines from the *mut* complementation group, which express mutations at the structural gene locus for the mutase apoenzyme, contained widely diverse amounts of CRM. Each of seven lines from the *mut*$^-$ subgroup, whose residual mutase activity reflects the presence of a structurally altered mutase protein with reduced affinity for cofactor, had detectable CRM ranging from 20% to 100% of control. The 21 lines from the *mut*^{0} group, which have no detectable mutase activity *in vitro*, fell into two populations with regard to CRM: 9 lines had detectable CRM ranging from 3% to 40% of control; 12 others had no detectable CRM (limit of detectability, <1% of control). These results emphasize the wide range of mutations at a single structural gene locus that can result in deficient enzyme activity.**

The methylmalonic acidemias are a group of genetically determined metabolic diseases resulting from deficiencies in the activity of the mitochondrial enzyme methylmalonyl-CoA mutase (2-methylmalonyl-CoA CoA-carbonylmutase, EC 5.4.99.2; called herein "mutase") (1). Mutase catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA and requires adenosylcobalamin (AdoCbl) as a cofactor. Mutase is important in the pathway by which odd-chain fatty acids, branched-chain amino acids, thymine, uracil, and cholesterol are metabolized.

Previous biochemical and genetic studies on skin fibroblasts from affected individuals have shown that two general bases for mutase deficiency exist (2). In fibroblasts from some patients, intracellular synthesis of AdoCbl from its precursor, hydroxocobalamin (vitamin B12), is defective, resulting in reduced mutase activity *in vivo*. In cell-free extracts of fibroblasts from such patients, however, mutase activity is normal when assayed with excess added cofactor. These mutants in cobalamin (Cbl) metabolism have been divided into four groups (*cbl A–D*) by complementation analysis with polyethylene glycol-induced heterokaryons (3).

In fibroblasts from other patients, Cbl metabolism is normal, and it is the mutase apoenzyme itself which is defective (4). These cell lines form a single complementation group, *mut*. Biochemical studies in cell-free extracts of fibroblasts from this group have shown that many of them contain no detectable mutase activity under any conditions of assay; this subgroup is designated *mut*^{0} (5, 6). Several other *mut* lines, however, have residual mutase activity that can be detected when large amounts of AdoCbl (>500 times the normal K_m) are added to the assay. These lines, called *mut*$^-$, have been shown to contain mutase enzymes that have abnormally high K_m values for AdoCbl (5, 6). In addition, the mutase enzymes in most of these *mut*$^-$ mutants have reduced V_{max} values when compared to control.

The above data on fibroblasts from the *mut* complementation group establish the fact that the abnormalities in these lines result from mutations at the structural gene locus for mutase. The molecular bases for these mutations remains obscure, however, particularly for those cell lines in the *mut*^{0} subgroup. To provide additional insight on this question from a different experimental perspective, we have developed a radioimmunoassay (RIA) to quantitate the amount of immunochemically crossreacting mutase protein present in extracts of normal fibroblasts and of fibroblasts from the *mut* complementation group. We report here the results of these studies on 28 *mut* lines (21 *mut*^{0}, 7 *mut*$^-$), 4 *cbl* mutant lines, and 4 normal lines.

MATERIALS AND METHODS

Cells and Cell Culture. Human skin fibroblasts were routinely maintained in 75 cm^2 flasks in Eagle's minimal essential medium, supplemented with 10% (vol/vol) fetal calf serum as described (7). Cells were harvested with 0.25% trypsin-EDTA (7) and were washed three times at 4°C with 10 ml of 0.15 M NaCl/0.01 M potassium phosphate, pH 7.5. The cell pellets were stored frozen at −90°C until used. The group and subgroup designations of the mutant lines were determined by complementation analysis (3) and by direct assay of mutase activity (ref. 6; A. Hack, personal communication).

Preparation of Fibroblast Extracts. Each frozen cell pellet (containing the cells from one flask) was dispersed in 1 ml of 0.15 M NaCl/0.01 M potassium phosphate, pH 7.5, and sonicated in an ice bath with the microprobe of a Bronson sonicator for 5 sec at a setting of 2 (approximately 50 W). Sonication was repeated four times at 1-min intervals for each of the samples, and then they were centrifuged at 1700 × *g* for 15 min at 4°C. The supernatant fluid was recovered and immediately used in the RIA.

Purification and Iodination of Human Mutase. Mutase was purified to homogeneity from human placenta as described (8). One ml of 0.01 M Tris·HCl, pH 8/0.125 M NaCl/0.002 M re-

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Abbreviations: Cbl, cobalamin; AdoCbl, adenosylcobalamin; RIA, radioimmunoassay; CRM, crossreacting material.

duced glutathione/100 μg of protein was dialyzed against 4 liters of 0.15 M NaCl/0.01 M potassium phosphate, pH 7.5, for 7 days at 4°C, with a change each day. The final dialysis was against 0.015 M NaCl/0.01 M borate·KOH, pH 8.5 (two changes). The solution was concentrated to 0.1 ml under a stream of nitrogen at 4°C.

One mCi (1 Ci = 3.7×10^{10} becquerels) of ^{125}I-labeled *p*-hydroxyphenylpropionic acid, *N*-hydroxysuccinimide ester (4000 Ci/mmol; New England Nuclear) was dried at 4°C under a stream of nitrogen. Of the concentrated protein, 15 μg was added to the ester, and the iodination was completed as described (9). The iodinated protein was separated from unreacted ^{125}I-labeled ester by gel filtration on a 2 × 90 cm column of Sephadex G-150 equilibrated and eluted with 0.25 M NaCl/0.01 M potassium phosphate, pH 7.5/2.5 mg of porcine gelatin per ml (9). This procedure resulted in the formation of ^{125}I-labeled mutase which had a specific activity of approximately 1×10^6 cpm per μg of protein.

RIA. A chicken was immunized with purified human mutase, and antiserum was obtained as described (8). The RIA used 0.7 ml containing 35 μmol of potassium phosphate (pH 7.5), 150 μmol of NaCl, various amounts of purified unlabeled mutase or fibroblast extract, and 0.1 ml of serum consisting of antiserum diluted with preimmune serum. This mixture was incubated for 24 hr on a shaking platform at 4°C. Then 12 ng of ^{125}I-labeled mutase was added, and the mixture was incubated again for 24 hr on a shaking platform at 4°C. Subsequently, 0.3 ml of saturated ammonium sulfate (Tris-buffered, pH 8) was added to each assay. The samples were mixed, incubated for 10 min at 4°C, and then centrifuged at 30,000 × *g* for 30 min. Aliquots of 0.75 ml were removed from the supernatants, and their content of ^{125}I was determined in a Beckman Gamma 8000 system at 45% efficiency. Each set of assays contained controls in which no antiserum was added and others in which no unlabeled mutase or fibroblast extract was added. The final concentration of 30% ammonium sulfate resulted in the precipitation of 10–20% of the ^{125}I-labeled mutase in the presence of preimmune serum. A standard curve was constructed for each RIA with purified mutase (2–60 ng). The limit of detectability was 2 ng of added mutase. Each fibroblast line was assayed at least twice, replicate values agreeing within 10% in each case. Samples were initially screened by using 0.4 ml of the fibroblast extract in the RIA. For a number of lines, the crossreacting material (CRM) in this amount of extract exceeded the upper limits of linearity of the standard curve of the RIA (see Fig. 3). These samples were reassayed with smaller quantities of extract so that CRM was within the range of 7.5–15.0 ng. Other lines containing small quantities (<2.4 ng/0.4 ml) of CRM were reassayed with extracts prepared from 3 times as many cells. If no increase in CRM was observed, the line was considered CRM negative.

Anion Exchange Chromatography. A normal cell line and mutant cell line 454 (two 75-cm^2 flasks each) were grown and harvested. After a final wash with 10 ml of 0.02 M sodium phosphate (pH 7.0), the cells were suspended in 2 ml of this buffer and sonicated. Each extract was applied to a 0.7 × 3.0 cm column containing 0.5 ml of DEAE-cellulose (Whatman) equilibrated with the same buffer, and the columns were washed with 10 ml of the buffer. Then the protein was eluted with a linear gradient (total volume, 60 ml) from 0.02 M sodium phosphate (pH 7) to 0.4 M NaCl/0.02 M sodium phosphate, pH 7. Fractions of 0.6 ml were collected, and 0.4 ml of each fraction was assayed for CRM by the RIA. Human placenta was solubilized in 0.028 M sodium phosphate (pH 7.0) as described (8) and fractionated on DEAE-cellulose as described, except on a larger scale. Fractions were collected and assayed for mutase activity in the presence of 60 μM AdoCbl as decribed (8) and for CRM by RIA.

Protein Determination. Protein was determined as described (10) with bovine serum albumin as a standard.

Polyacrylamide Disc Gel Electrophoresis. Polyacrylamide disc gel electrophoresis and protein staining were as described (8). Gels run simultaneously were cut into 2-mm slices, which were either assayed for ^{125}I content or eluted (8) and assayed for mutase activity.

RESULTS

Purification and Iodination of Mutase. Fig. 1 shows the mobility of purified mutase and ^{125}I-labeled mutase on polyacrylamide disc gel electrophoresis. A single peak of ^{125}I activity was observed which corresponded to the peak of mutase activity and to the stained band in two parallel gels. In addition, when ^{125}I-labeled mutase was applied to a G-150 Sephadex column and eluted, only a small quantity of bound radioactivity appeared in the void volume, whereas 90% of the bound ^{125}I was eluted as a single symmetrical peak at 1.25 times the void volume, the typical elution volume for mutase (11) (data not shown).

Radioimmunoassay. Studies (8) with the antiserum utilized here have shown that it was able to inhibit mutase enzyme activity. In order to determine its efficacy in precipitating the ^{125}I-labeled mutase, the experiment shown in Fig. 2 was carried out. Increasing amounts of antiserum precipitated increasing amounts of ^{125}I-labeled mutase up to ≈95% of the added label. The amount of the antiserum required to precipitate 1 μg of the labeled protein (25 μl) was similar to that reported to be necessary

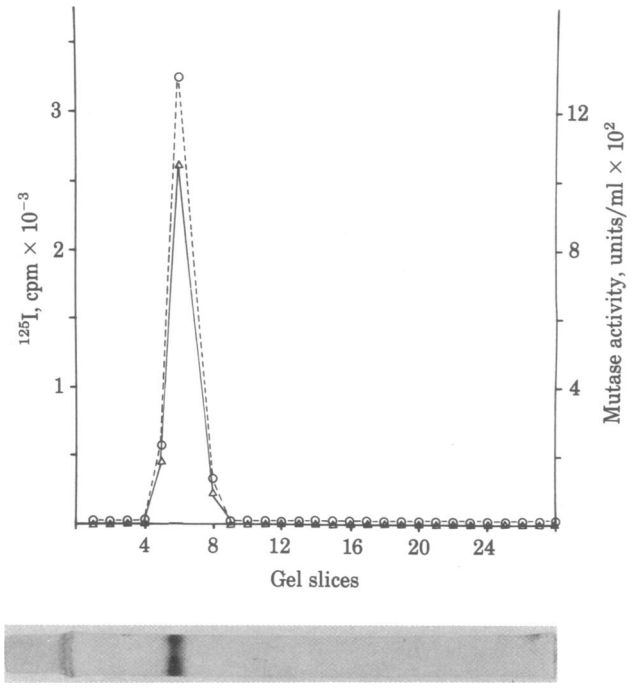

FIG. 1. Polyacrylamide disc gel electrophoresis of purified human mutase and iodinated mutase. Samples containing 15 μg of purified human mutase were subjected to electrophoresis on parallel polyacrylamide gels (total monomer, 7%) at 4°C. (*Lower*) One gel was stained for protein with Coomassie blue. (*Upper*) The second was sliced, the slices were eluted, and mutase activity was determined as described (8) (△——△); one unit equals one μmol of product per min. A small amount of ^{125}I-labeled mutase (6 ng) was subjected to electrophoresis on a third gel. The gel was sliced, and the ^{125}I content of the slices was determined (○– – –○).

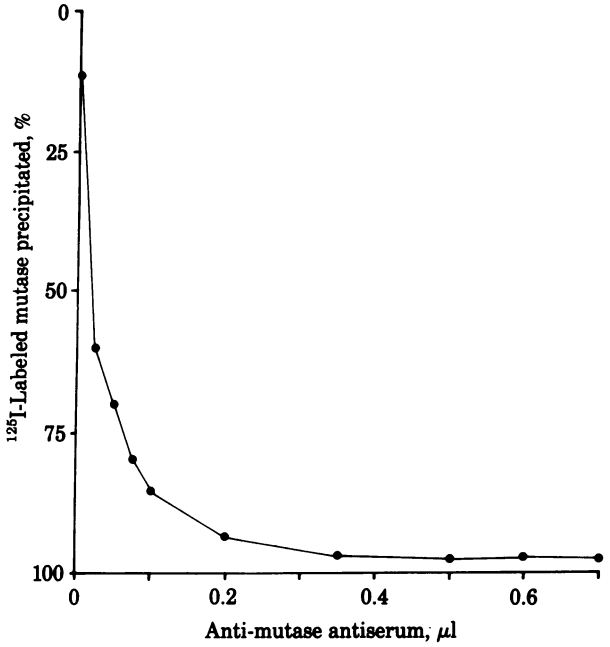

FIG. 2. Precipitation of ^{125}I-labeled mutase by anti-mutase antiserum. ^{125}I-Labeled mutase (12 ng) was incubated for 24 hr at 4°C with 0.1 ml of chicken serum (consisting of the indicated volume of anti-mutase antiserum diluted with preimmune serum), 35 μmol of potassium phosphate (pH 7.5), and 150 μmol of NaCl in a total volume of 0.7 ml. The immune complexes were precipitated and their ^{125}I content was determined. The ordinate shows the percentage of added ^{125}I present in the precipitated material.

for the precipitation of purified mutase (33 μl) (8).

The RIA was based on the principle of competition between unlabeled mutase and ^{125}I-labeled mutase for a limiting quantity of antibody. Routinely, an amount of antiserum was added that was sufficient to precipitate 70–80% of the label in the absence of unlabeled mutase. Fig. 3 is a typical standard curve that shows the ability of increasing quantities of unlabeled, purified mutase to inhibit the precipitation of ^{125}I-labeled mutase; the amount of ^{125}I in the supernatant in the absence of unlabeled

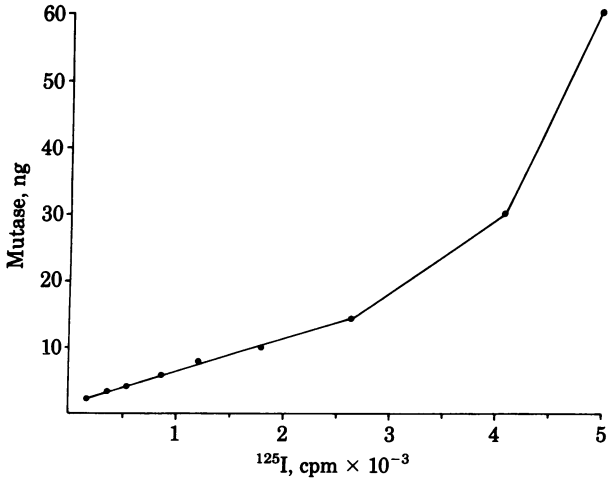

FIG. 3. Standard curve for the RIA of mutase CRM. Increasing amounts of unlabeled purified mutase were incubated with 0.05 μl of anti-mutase antiserum and ^{125}I-labeled mutase (12 ng; 12,000 cpm). After precipitation of the immune complex, the ^{125}I content of an aliquot of the supernatant from each sample was determined. These values were reduced by the amount of ^{125}I detected in the supernatant in the absence of unlabeled mutase (2700 cpm).

mutase was subtracted from the values on the abscissa. Approximately 14 ng of unlabeled mutase was required to inhibit the precipitation of 12 ng of ^{125}I-labeled mutase by 50%, whereas nearly complete inhibition required 60 ng of mutase. These studies suggest that the antigenicity of the ^{125}I-labeled mutase differs little from that of the unlabeled purified mutase and establish its suitability for use in the RIA.

RIA of Fibroblast Lines. Table 1 contains the results of the RIA of extracts from control cell lines and from 32 mutant fibroblast lines from patients with inherited methylmalonic acidemia. The conditions of storage, washing of cells, and extraction of mutase were identical for all cell lines. Control cells contained 193–297 ng of CRM per mg of cell protein (mean, 237 ng of CRM per mg). All cell lines of the *cbl A–D* groups, which are defective in intracellular Cbl coenzyme synthesis, had normal amounts of CRM. On the other hand, the mutase apoenzyme mutants had varying quantities of CRM. The seven *mut*$^-$ mutants each had detectable CRM ranging from 20% to 100% of control. The CRM in the *mut*^{0} cell lines was undetectable in

Table 1. Immunochemically CRM in control fibroblasts and in fibroblasts from patients with inherited methylmalonic acidemia

Complementation group	Yale cell line number	CRM,* ng/ml	Cell protein, mg/ml	CRM/ protein, ng/mg
Control	82	104	0.350	297
Control	237	90	0.400	225
Control	86	144	0.710	203
Control	87	160	0.830	193
cbl B	283	45	0.140	322
cbl A	17	37	0.160	231
cbl C	792	36	0.175	206
cbl D	414	96	0.630	152
mut$^-$	601	208	0.830	251
mut$^-$	378	130	1.240	105
mut$^-$	257	172	2.260	76
mut$^-$	394	65	0.860	76
mut$^-$	589	35	0.470	73
mut$^-$	515	49	1.000	49
mut$^-$	550	34	0.820	42
mut^0	454	64	0.685	93
mut^0	354	8.5	0.095	90
mut^0	438	96	1.300	74
mut^0	507	15	0.215	70
mut^0	417	16	0.290	55
mut^0	663	12	0.490	25
mut^0	418	14	0.820	17
mut^0	471	18	1.790	10
mut^0	437	13	1.570	8
mut^0	77	<6	1.080	—
mut^0	184	<6	0.360	—
mut^0	216	<6	1.010	—
mut^0	294	<6	3.450	—
mut^0	296	<6	0.370	—
mut^0	379	<6	0.200	—
mut^0	444	<6	1.360	—
mut^0	460	<6	0.415	—
mut^0	464	<6	0.260	—
mut^0	552	<6	1.540	—
mut^0	588	<6	1.360	—
mut^0	505	<6	0.515	—

Material crossreacting immunochemically with human methylmalonyl-CoA mutase was determined in extracts of fibroblasts by RIA. Cell protein was determined in the same extracts.

* The limit of sensitivity of the RIA is 6 ng/ml. Each of the lines with undetectable CRM was assayed again with 3 times more cell protein; no CRM was detected.

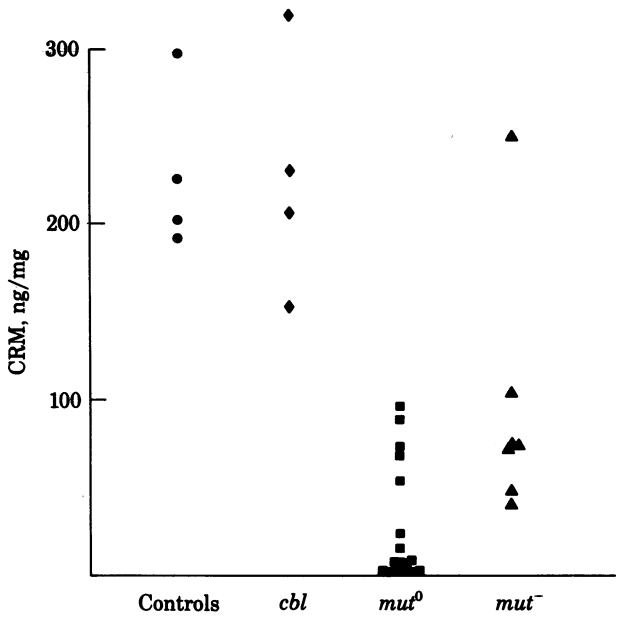

FIG. 4. CRM in extracts of control fibroblast lines and lines from the *cbl*, *mut*^{0}, and *mut*$^-$ groups.

12 lines and ranged from ≈3% to 40% of control in 9 others (Fig. 4). Significantly, replicate assays in each of the nine *mut*^{0} lines with CRM differed by <10%. Moreover, selected *mut*$^-$ and *mut*^{0} lines were tested for CRM by an independent immunoassay involving inhibition of the precipitation of mutase activity by antiserum. The amounts of CRM detected were comparable to the quantities found by RIA (data not shown).

Line 454, the *mut*^{0} mutant with the largest amount of CRM, was studied further by anion exchange chromatography (Fig. 5). The elution profile of the CRM from the mutant line (Fig. 5C) was essentially identical to that obtained for CRM from a control line (Fig. 5*B*) and from human placenta (Fig. 5*A*), suggesting that the mutant protein differed very little in total charge from normal mutase.

DISCUSSION

The RIA developed in the course of these studies has proved to be both specific and sensitive. The ^{125}I-labeled human mutase produced by the Bolton–Hunter procedure comigrates with unlabeled mutase on polyacrylamide gel electrophoresis (Fig. 1) and Sephadex chromatography. It interacts with antiserum raised against the purified human enzyme at approximately the same titer as the unlabeled enzyme, is precipitated by the antiserum to an extent exceeding 90% (Fig. 2), and competes with the unlabeled enzyme for binding to the antibody. The RIA itself is linear over a range of 2–15 ng of added unlabeled antigen (Fig. 3) and detects a single peak of CRM (which coincides with the elution position of mutase activity) when applied to fractions from DEAE-cellulose column chromatography of crude placental or control fibroblast extracts (Fig. 5).

The data in Table 1 and Fig. 4 on the quantity of CRM detected by this RIA in extracts of fibroblasts show the expected diversity both within and among the mutant groups tested. The range of values obtained for control lines is relatively small; mutants of the *cbl* mutant groups have amounts of CRM that are not different from that of control, as expected on the basis of their normal mutase activity *in vitro* (2).

The 7 *mut*$^-$ mutants, which are AdoCbl K_m mutants, all show residual crossreacting material, ranging from 20% to 100% of control. The molecular lesions in these mutants are most likely

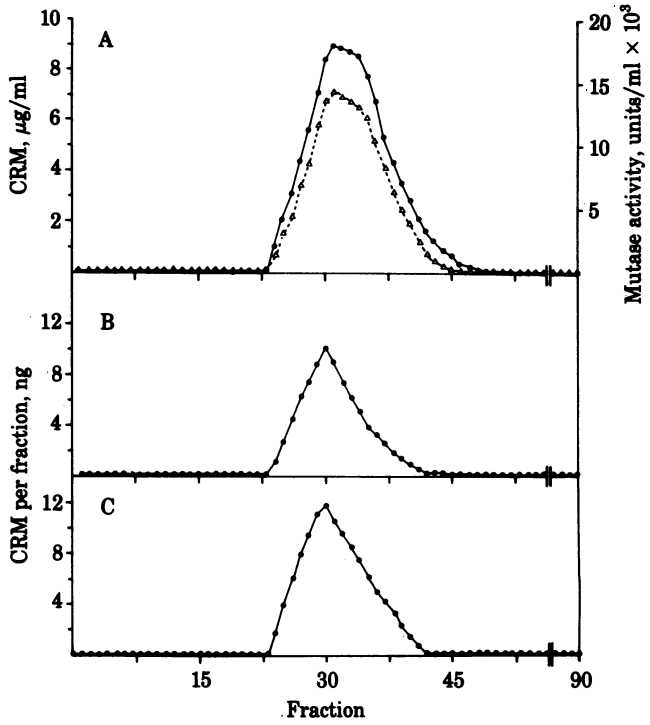

FIG. 5. DEAE-cellulose chromatography of mutase activity and CRM in extracts from human placenta (*A*), control fibroblasts (*B*), and fibroblasts from a *mut*^{0} mutant (line 454) (*C*). The NaCl gradient was started at fraction 1. Mutase activity (△---△ ; one unit equals one μmol of product per min) and CRM (●——●) were determined as before. In *B*, 0.8 mg of protein was applied to the column, and 52% of the applied CRM was recovered in the column fractions; in *C*, 1.5 mg of protein was applied, and 60% of the CRM was recovered.

single base changes which lead to amino acid substitutions that affect the kinetic parameters of the enzyme. In six of the seven lines reported here, these changes result simultaneously in fewer enzyme molecules being present in the cell because of either lowered rates of synthesis or increased rates of degradation. In the other line, the synthesis and degradation rates appear not to be affected. Similar findings have been reported for fibroblasts from patients with cystathionine synthase deficiency, which contain residual enzyme activity and CRM for synthase (12).

The amount of CRM in the *mut*$^-$ lines does not correlate well with the residual catalytic activity, as measured by the V_{max} values obtained from Lineweaver–Burk plots by Willard and Rosenberg (6) and Willard (13). Table 2 presents a comparison of the V_{max} values and CRM in these lines. Five of the lines have less residual catalytic activity than CRM; however, the other two have 2 and 3 times more catalytic activity per unit CRM than control has. The explanation for the latter phenomenon may be that the mutation in these two lines results in the production of a protein both with significantly lowered affinity for antibody and with abnormal reaction kinetics. Other explanations, including the possibility of structural alterations in the mutase protein that lead to increased catalytic activity per enzyme molecule, seem less likely, although a precedent for such mutations exists. Affected members of one family, whose erythrocytes show a 2- to 3-fold increase in the activity of phosphoribosyl pyrophosphate synthase, have been reported to have a normal amount of CRM for the synthase protein and, hence, a 2- to 3-fold increase in the specific activity of the enzyme (14).

The *mut*^{0} mutants fall into two groups (Table 1, Fig. 4): those with CRM (9 lines) and those without detectable CRM (12 lines). The CRM positive lines may be viewed as more extreme

Table 2. Comparison of V_{max} and CRM in extracts of control fibroblasts and fibroblasts of the *mut*$^-$ group

Yale cell line	V_{max},* units/mg	CRM,† ng/mg	V_{max}/CRM, unit/ng
Control			
87	1672	193	8.66
237	1804	225	8.02
82	1499	297	5.05
mut$^-$			
550	725	42	17.3
515	602	49	12.3
257	291	76	3.8
394	77	76	1.0
378	18	105	0.17
601	38	251	0.15
589	7	73	0.10

* V_{max} values from Willard and Rosenberg (6) and Willard (13). One unit of enzyme activity is defined as one pmol of product formed per min.

† CRM data was taken from Table 1.

cases of the *mut*$^-$ lines. Again, single base and amino acid changes seem the most likely molecular defects; in the *mut*^{0} lines, these produce mutase proteins with no catalytic activity and result in significant reductions in the steady-state level of antigen. Other explanations can be invoked to account for the 12 CRM negative lines, however. While single base changes and amino acid substitutions leading to undetectable amounts of mutase protein are possible, nonsense and frame-shift mutations are more likely. In addition, mutations in a regulatory gene or in DNA- or RNA-processing sites within the mutase structural gene could account for the absence of both CRM and catalytic activity. Finally, it is possible that the mutant enzymes in these cell lines are so altered that they are not recognized by antibodies raised against the normal enzyme. Studies capable of probing the intimate structure of the mutase gene and mRNA will be required to differentiate these possibilities.

This series of 28 human fibroblast lines expressing mutations at a single locus is the largest yet tested by immunochemical techniques for the presence of CRM. Only two other studies have examined comparable numbers of mutant individuals: 20 fibroblast lines from patients with cystathionine synthase deficiency (12) and 18 patients with hypoxanthine phosphoribosyltransferase deficiency (15, 16). In the case of cystathionine synthase deficiency, immunologic heterogeneity similar to that already detailed was observed. Mutant fibroblasts with residual enzyme activity had variable amounts of CRM, whereas those without activity were subdivided into those with and those without detectable CRM (12). Other studies have not uncovered the same extent of diversity in amounts of CRM as these two reports have. For example, hemolysates from all 17 patients with complete hypoxanthine phosphoribosyltransferase deficiency had no detectable CRM (15, 16). The one patient with significant residual enzyme activity was shown to be a K_m mutant and had detectable CRM (15, 16). CRM was similarly absent in patients with complete deficiency of muscle phosphorylase (17), sucrase/isomaltase (18), and purine nucleoside phosphorylase (19), whereas three patients with total galactose-l-phosphate uridyltransferase deficiency had normal amounts of CRM in erythrocytes (20). The small number of patients reported in most of these studies may account for the lack of heterogeneity observed, although other explanations will have to be sought if further work produces similar results.

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