INHERITED DEFICIENCIES OF HUMAN METHYLMALONYL CoA MUTASE ACTIVITY: REDUCED AFFINITY OF MUTANT APOENZYME FOR ADENOSYLCOBALAMIN

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SUMMARY: We have studied the affinity of methylmalonyl CoA mutase for its required cofactor, adenosylcobalamin, in extracts of control and mutant human cultured fibroblasts. Control enzyme has an apparent K_m for adenosylcobalamin of 6-7 x 10^{-8} M. Five mutant cell lines from patients with methylmalonicacidemia due to a mutase apoenzyme defect were studied. Three have undetectable mutase activity (<0.15% of control) at all cofactor concentrations. Two others, however, have markedly altered K_m's for adenosylcobalamin of 2.8 x 10^{-4} M and 1.7 x 10^{-5} M. These mutant lines synthesize adenosylcobalamin normally and, by complementation analysis, are genetically identical to all other mutase apoenzyme mutants tested. We conclude that the mutase deficiency in these two cell lines results from structurally altered mutase apoenzymes with markedly reduced affinities for adenosylcobalamin.

Inherited deficiencies of methylmalonyl CoA mutase activity (EC 5.4.99.2) in man comprise at least five biochemically and genetically distinct mutant classes (1-3), four characterized by deficient synthesis of adenosylcobalamin (AdoCbl)*, the essential Cbl coenzyme required for mutase activity, and one characterized by an apparently mutant or absent apoenzyme (4-6). Cultured human fibroblasts belonging to this fifth mutant class metabolize Cbl normally (5-8), are severely deficient in mutase activity in cell extracts even at normally saturating concentrations of AdoCbl *in vitro* (7), and constitute a distinct genetic complementation group, designated *mut* (2). The present studies were initiated to elucidate the biochemical and genetic nature of the defect in a series of presumptive apoenzyme mutants. We present enzymatic evidence that two cell lines in this series are each characterized by an abnormal mutase apoenzyme with markedly reduced affinity for AdoCbl. These data provide a direct demonstration that the *mut* complementation group reflects

*Abbreviations: AdoCbl, adenosylcobalamin; Cbl, cobalamin; OH-Cbl, hydroxocobalamin.

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mutations at the structural gene locus for the methylmalonyl CoA mutase apoenzyme.

MATERIALS AND METHODS

Cell culture. Human skin fibroblasts were routinely maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, as described (1). Intact cells were assayed *in situ* for their ability to incorporate ^{14}C from [1-^{14}C]propionate into acid-precipitable material as described (9). Genetic complementation analysis was performed as described (2) in polyethylene glycol-induced heterokaryons using complementation panels. To measure synthesis of Cbl coenzymes, fibroblasts in monolayer were incubated with OH-[^{57}Co] Cbl for 7 days (2); cobalamins were then isolated and identified by the method of Fenton and Rosenberg (10).

Assay of methylmalonyl CoA mutase activity. Methylmalonyl CoA mutase activity in cell extracts was determined radioisotopically by measuring the production of [^{14}C]succinate from DL-[methyl-^{14}C]methylmalonyl CoA. Cells were harvested with 0.25% trypsin-EDTA and centrifuged at 600 x g for 5 min. Extracts were prepared by resuspending cells in 0.14 M NaCl, 0.003 M KCl, 0.01 M phosphate buffer at pH 7 and incubating them for 30 min at 4° in Lubrol WX (0.3 mg/mg protein) under reduced illumination. All further operations involving cell extracts were performed in the dark. After Lubrol treatment, the cell suspension was centrifuged at 12,000 x g for 15 min and the supernatant used directly as a source of enzyme. The reaction mixture contained (in a final volume of 180 μl): 200 mM Tris-sulfate, pH 7.5; 0.42 mM DL-[methyl-^{14}C]methylmalonyl CoA (New England Nuclear Corp.; final specific activity, 2.6 mCi/mmol); 0.1-1.0 mg cell extract protein; and AdoCbl as indicated. When AdoCbl was added, cell extracts were preincubated with it for 10 min at 37°. Reactions were initiated by addition of methylmalonyl CoA, allowed to proceed for 7 min at 37°, and terminated by the addition of 100 μl of 6 N H_2SO_4. Samples were placed in a boiling water bath for 5 min and [^{14}C]succinate extracted with ether as described (11). The ether phase was brought to dryness, and samples were applied to Whatman 3 MM chromatography paper with 100 nmol authentic succinate and methylmalonate. Chromatograms were developed 12-16 hr with a solvent consisting of ethanol:H_2O:conc. NH_4OH (80:15:15). The positions of succinate and methylmalonate were located with 0.04% bromocresol green. With this solvent system, counts co-chromatographing with succinate migrate 2-3 cm ahead of those with methylmalonate; resolution of [^{14}C]succinate from [^{14}C]methylmalonate is greater than 99%. Radioactivity was determined using a Packard liquid scintillation spectrometer with Liquifluor-toluene as scintillation fluid. Mutase activity is expressed as pmol succinate formed/min/mg cell protein. Protein content was determined by the Fluram method (12).

RESULTS

The five mutant cell lines employed in this study were originally characterized as apoenzyme mutants by direct assay of mutase activity. When measured in the absence of added AdoCbl *in vitro*, each had undetectable activity (<2 pmol/min/mg). In the presence of 5 x 10^{-6} M AdoCbl, four lines had undetectable activity and one had an activity of 8 pmol/min/mg. Control cell lines have activities of 18-56 (mean 25) pmol/min/mg in the absence of added AdoCbl

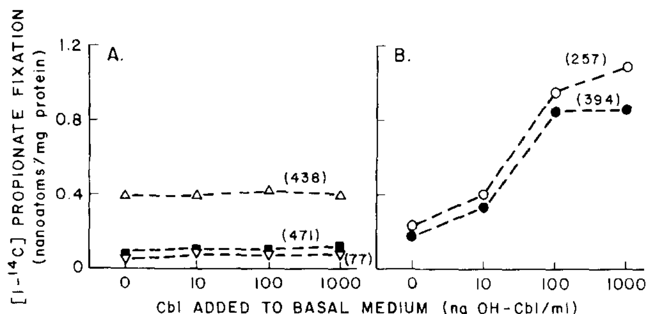

Figure 1. Incorporation of ^{14}C from [1-^{14}C]propionate into acid-precipitable material by intact human cultured fibroblasts. Cells were grown for 2-3 days in basal medium or in medium supplemented with 10, 100, or 1000 ng OH-Cbl/ml and assayed (9). A. Three unresponsive apoenzyme mutants. B. Two responsive apoenzyme mutants. Under the same conditions, control cells fix >3.0 nanoatoms/mg. Numbers in parentheses refer to the laboratory identification number for each cell line.

and 898-1173 (mean 1341) pmol/min/mg in the presence of 5×10^{-6} M AdoCbl, indicating that, under these growth conditions, approximately 98% of the enzyme exists as apomutase.

As part of a larger study assessing mutase activity in mutant cell lines after growth in Cbl-supplemented medium, we grew the five apoenzyme mutant lines up to 30 days in basal medium containing 30-50 pg Cbl/ml, and in medium supplemented with 10, 100, or 1000 ng OH-Cbl/ml. Mutase activity in intact cells was monitored by the fixation of [1-^{14}C]propionate _in situ_ (9). Three cell lines (77, 438, and 471) (Fig. 1A) were markedly deficient in ^{14}C fixation under basal conditions and were completely refractory to Cbl supplementation, even after 30 days. Two other lines (257 and 394), however, were deficient when grown under basal conditions, but displayed significant increases in ^{14}C fixation when grown for 2-3 days in Cbl-supplemented medium (Fig. 1B); no further increases were observed upon growth for longer periods of time.

The observation that some presumptive apoenzyme mutants are responsive to Cbl supplementation in culture led us to examine the effect of AdoCbl concentration on the reaction kinetics of both control and mutant methylmalonyl CoA mutase in fibroblast extracts. The data indicate that the mutase-AdoCbl inter-

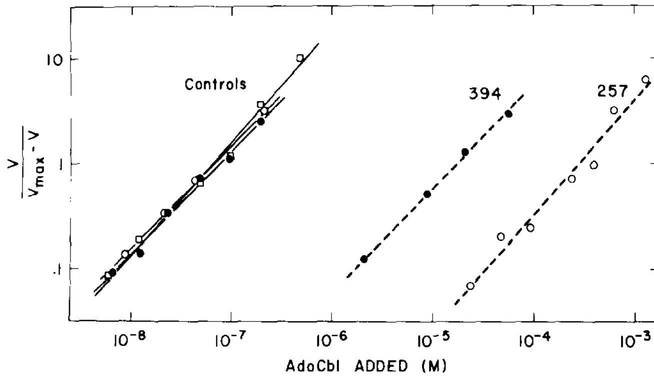

Figure 2. Hill plots of the effect of AdoCbl concentration on methylmalonyl CoA mutase activity in extracts of control and mutant human cultured fibroblasts. Kinetic parameters derived from these data are listed in Table 1. Solid and dotted lines were fit by least squares regression analyses. Control cell lines: 82 (●——●); 87 (o——o); and 105 (□——□). Mutant cell lines: 257 (o- - -o); and 394 (●- - -●).

action follows simple Michaelis-Menten kinetics characterized by non-cooperative binding of AdoCbl. Figure 2 presents Hill plots showing the effect of AdoCbl concentration on reaction velocity. The Michaelis constants for AdoCbl (K_m AdoCbl) and the maximum velocities (V_{max}) for three control lines and the five apoenzyme mutants are listed in Table 1. Control enzyme has an apparent K_m AdoCbl of 6-7 x 10^{-8} M, in good agreement with previous studies of both crude fibroblast preparations (13,14) and the purified sheep liver enzyme (15). The three unresponsive mutants (Fig. 1A) had no detectable activity (<0.15% of the control mean) even at 6 x 10^{-4} M AdoCbl. As shown in Fig. 2, however, kinetic studies on extracts of responsive cell lines 257 and 394 revealed markedly elevated K_m's for AdoCbl of 2.8 x 10^{-4} M and 1.7 x 10^{-5} M, respectively. Their apparent V_{max}'s were also abnormal, being approximately 20% and 5% of control, respectively (Table 1).

To rule out the possibility that lines 257 and 394 have defects in Cbl metabolism, we measured [^{57}Co]Cbl accumulation and AdoCbl synthesis by intact cells in culture. Cell lines 257 and 394 accumulated 302 and 192 pg [^{57}Co]Cbl/mg protein and converted 11% and 6% of it to Ado[^{57}Co]Cbl,

Table 1. Kinetic parameters of methylmalonyl CoA mutase from control and mutant human cultured fibroblasts

Cell line	K_m AdoCbl* (10^{-8} M)	V_{max} * (pmol/min/mg)
Controls		
82	7	1053
87	6	1770
105	7	1333
Mutants		
257	28,000	291
394	1,700	74
77	**	<2
438	**	<2
471	**	<2

* Apparent K_m AdoCbl and V_{max} were derived from plots of 1/v vs. 1/[AdoCbl].

** K_m AdoCbl could not be determined on these cell lines because activity was undetectable (<2 pmol/min/mg) at all concentrations.

respectively. The corresponding figures for two control lines were 207 and 361 pg [^{57}Co]Cbl/mg protein accumulated and 22% and 13% converted to Ado[^{57}Co]Cbl. These data are consistent with previous studies of AdoCbl synthesis in control and apoenzyme mutant cell lines (7). Cells with known defects in AdoCbl synthesis converted <0.5% to Ado[^{57}Co]Cbl. Synthesis of methylcobalamin, the other Cbl coenzyme, was also normal in lines 257 and 394.

Further, we performed genetic complementation analysis using complementation panels of polyethylene glycol-induced heterokaryons (2). As shown in Table 2, the deficiencies in lines 257 and 394 complemented the four known defects in Cbl metabolism (designated *cbl A*, *cbl B*, *cbl C*, and *cbl D* in refs. 1, 2), but did not complement the defect characterizing the *mut* complementation

Table 2. Genetic complementation analysis in polyethylene glycol-induced heterokaryons of human cultured fibroblasts

	Change in ^{14}C fixation when fused to:*					
Cell line	self	cbl A	cbl B	cbl C	cbl D**	mut
77	-.05	1.63	.86	.92	.86	-.03
257	-.02	1.62	1.34	.96	.85	-.03
394	.03	1.22	1.56	1.59	1.18	.00

* Cell lines were mixed 1:1 with representatives of each complementation group (1,2), fused with polyethylene glycol, and assayed for [^{14}C]propionate fixation 18-32 hr later (2,9). Data represent differences in fixation between fused and unfused, but otherwise identical, samples and are expressed as nanoatoms ^{14}C fixed/mg protein in 10 hr. Mutant cell lines alone incorporate 0.07-1.09 nanoatoms/mg.

** A newly described complementation group (2,3).

group. In this respect, lines 257 and 394, as well as lines 438 and 471 (data not shown), are similar to line 77 (Table 2), which was obtained from the first described patient with a putative apoenzyme defect (4) and was used as a prototype for the mut complementation group (1).

DISCUSSION

A number of observations indicate that the mutase deficiency in each of the five mutant cell lines studied is due to a primary defect in the mutase apoenzyme. First, they accumulated Cbl and converted it to AdoCbl normally, in agreement with data on other presumptive apomutase mutants (7,8). Second, complementation analysis indicated that each of the five mutant lines could be assigned to the mut complementation group (Table 2), which is genetically distinct from the four known defects in Cbl metabolism. And third, all five lines were severely deficient in mutase activity measured in fibroblast extracts at normally saturating AdoCbl concentrations (Fig. 2). Three of the five lines had no detectable activity at AdoCbl concentrations up to 6 x 10^{-4} M. Two other lines expressed measurable, although still deficient, levels of activity

at very high AdoCbl concentrations *in vitro* (Table 1). The mutant enzyme from these two lines (257 and 394) demonstrated an approximately 4000- and 250-fold lower affinity for AdoCbl, respectively, than did control enzyme (Fig. 2). The demonstration that two cell lines belonging to this mutant class have biochemically defined defects in the binding of AdoCbl by methylmalonyl CoA mutase provides the first direct evidence that the *mut* complementation group is characterized by allelic mutations at the structural gene locus for the mutase apoenzyme.

Further, our data indicate that members of the *mut* complementation group comprise at least two subgroups within a genetically and biochemically distinct mutant class. Two patterns were observed when *mut* mutant cells were grown in Cbl-supplemented medium. Three cell lines did not respond at all with respect to mutase activity in intact cells (Fig. 1A), whereas the two mutant lines displaying abnormal K_m's for AdoCbl did respond significantly at Cbl concentrations greater than 10 ng/ml of culture medium (Fig. 1B). The precise relationship between this Cbl responsiveness in intact cells and measurements of mutase activity in cell extracts (Table 1) is not as yet clear. After five days of growth in medium containing 1000 ng OH-Cbl/ml, AdoCbl content in apoenzyme mutant cells increases 20-fold (8). Although no information exists on the intramitochondrial content of Cbl under these conditions, it is likely that the increase in activity in lines 257 and 394 reflects low levels of holomutase formation in the presence of very high concentrations of AdoCbl, overcoming the abnormally low affinity of the mutant enzyme for cofactor. The formation of holomutase may also serve to stabilize the mutant enzyme, thereby reducing the rate of degradation and increasing the amount of functional, although abnormal, mutase.

The nature of the defects in unresponsive apomutase mutants such as lines 77, 438, and 471 is not at all clear. Some may represent deletion mutations or single base changes such that no complete mutase gene product exists. Alternatively, a mutant apoenzyme may be present in these cells, but be un-

able to bind AdoCbl at any concentration or be severely deficient in a different part of the catalytic process involving the binding of methylmalonyl CoA or the intramolecular rearrangement of L-methylmalonyl CoA to succinyl CoA.

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