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Fibroblasts
methylenetetrahydrofolate reductase deficiency
mutation

Methylenetetrahydrofolate Reductase in Cultured Human Cells. II. Genetic and Biochemical Studies of Methylenetetrahydrofolate Reductase Deficiency

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Summary

Methylenetetrahydrofolate (methylene-H₄PteGlu) reductase activities in extracts of both normal and reductase-deficient cells were low and quite variable during the logarithmic phase of growth. Higher activities were detected reproducibly when the cultures were confluent. Methylene-H₄PteGlu reductase activities in extracts of fibroblasts from the parents of patient *CP* were about half of the level observed in normal control subjects. In fibroblasts from patient *CP*, the activity was 20% of normal whereas, from patients *LM* and *BM*, the activities were 19% and 14% of normal, respectively. When incubated at 55° in a solution containing all the components of the standard reaction mixture except the 5-methyl-H₄PteGlu substrate, the reductase activity in extracts of fibroblasts from two unrelated normal control subjects decreased to 31% and 22%, respectively, of the initial values after 30 min of incubation. In contrast, the reductase in extracts of cells patient *CP* was rapidly and exponentially inactivated at 55°. The reductase activity in extracts from patients *LM* and *BM*, the sisters, decreased to 22% and 38%, respectively, of the initial values. In repeated experiments the heat inactivation of reductase activity in extracts of cells from *LM* and *BM* closely resembled the normal control subjects in

total extent and time course of inactivation. The reductase activity in extracts from *WMA*, a fourth, unrelated patient was also completely inactivated but somewhat less rapidly than with patient *CP*. These results provide strong evidence that the reductase deficiency in these three families is due to different alleles. The data suggest that in *CP* and *WMA* there is a mutationally induced structural defect in the aporeductase as the basis for the observed alteration in thermostability, presumably reflecting reduced ability to bind the FAD cofactor.

Speculation

The observation that methylene-H₄PteGlu reductase activity in crude fibroblast extracts from two of the three families studied shows reduced thermal stability as well as decreased activity raises the possibility that this enzyme is unusually susceptible to mutationally induced alterations in cofactor binding.

Methylenetetrahydrofolate reductase deficiency is an inborn error of folate metabolism in which neurologic abnormalities are accompanied by moderate degrees of homocystinemia and homocystinuria but with normal or low plasma methionine

levels. The clinical findings in the three patients described in detail to date (3, 6, 9) have varied, but some degree of neurologic impairment has been consistent. More recently, methylene- H_4 PteGlu reductase deficiency has been suspected and the diagnosis confirmed in cultured fibroblasts from a fourth (2, 10) and a fifth (4) patient. The specific deficiency of methylene- H_4 PteGlu reductase was first identified in fibroblasts (6, 9) and confirmed in a postmortem liver specimen from one of the patients (4). Studies by Mudd *et al.* (6) suggested that the residual reductase activity in fibroblasts from patient *CP* was less stable when assayed without added flavin-adenine dinucleotide (FAD) than the reductase activities in cells from the other two patients, *BM* and *LM*, who are sisters. We report here studies of the residual methylene- H_4 PteGlu reductase activities in extracts of fibroblasts from all four patients. Our results suggest that the mutant reductase in each family is distinct. Furthermore, studies of cells from one patient and his parents suggest that methylene- H_4 PteGlu reductase deficiency is inherited in a Mendelian autosomal recessive pattern.

MATERIALS AND METHODS

Human skin fibroblasts were obtained from punch biopsy specimens and the cell lines were designated as follows: patient *CP*, MGF488; the parents of *CP*, MGF394 and MGF395; patient *LM*, MGF451; patient *BM*, MGF452; patient *WMa*, MGF548. The procedures used were reviewed and approved by the Committee on Human Studies of each institution, and included obtaining the informed consent of the patients and/or, where appropriate, the parents. The cells were grown in roller bottles containing 100 ml medium consisting of 85% Eagle's minimum essential medium (11) and 15% fetal calf serum (12), re-fed twice weekly. Methylene- H_4 PteGlu reductase activity in cell extracts was measured as described previously (7, 8).

RESULTS

Methylene- H_4 PteGlu reductase activities in extracts of both normal and reductase-deficient cells were low and quite variable during the logarithmic phase of growth. Higher activities were detected reproducibly when the cultures had reached confluency. Therefore, measurements were made on cultures which were confluent and had been harvested 2 days after re-feeding with fresh media. Methylene- H_4 PteGlu reductase activities in extracts of fibroblasts from the parents of patient *CP* were about half of the level observed in normal control subjects (Fig. 1). Fibroblasts from 10 unrelated normal control subjects yielded a mean value of 8.6 nmol formaldehyde formed per mg protein with a standard deviation of 1.4. The reductase activity in extracts of fibroblasts from patient *CP*'s father was 40% of the normal mean while the mother's value was 35% of normal. In

Fig. 1. Methylenetetrahydrofolate reductase activity in fibroblasts from normal control subjects, parents and patients. Each point represents cells from a separate individual and is the average of specific activities obtained in at least duplicate determinations differing by not more than 20%. HCHO: formaldehyde.

fibroblasts from *CP*, the reductase activity was 20% of normal, whereas from *LM* and *BM* the activities were 19% and 14% of normal, respectively. When equal amounts of extracts from normal and reductase-deficient cells were mixed, the total reductase activity was the sum of that in the individual cell extracts, suggesting that the reductase deficiency is not due to the presence of an inhibitor.

The pH activity profiles of reductase activity in extracts of cells from *CP* and *LM* gave relatively narrow curves with maximal activities near pH 6.3 as in the control subjects (data not shown). Preliminary kinetic studies of reductase activity in the crude extracts showed no consistent alterations in the apparent K_m for 5-methyl- H_4 PteGlu in cells from *CP* and *LM*. However, the low reductase activities in the reductase-deficient cells and the variability of the measured activities at low substrate concentrations precluded more precise studies.

The thermal stabilities of methylene- H_4 PteGlu reductase activity from the deficient cells were compared to normal subjects (Fig. 2). When incubated at 55° in a solution containing all components of the standard reaction mixture except the FAD cofactor and the 5-methyl- H_4 PteGlu substrate, the reductase in fibroblast extracts was almost entirely inactivated within 5 min (Fig. 2A), the reductase from the normal subjects being less rapidly inactivated than the residual reductase from the patients' cells. However, when incubated under these conditions but in the presence of 72 μ M FAD, varying degrees of cofactor stabilization were clearly seen (Fig. 2B). The reductase activity in extracts of fibroblasts from two unrelated normal control subjects decreased to 31% and 22%, respectively, of the initial values after 30 min of incubation. In contrast, the reductase in extracts of cells from patient *CP* was rapidly and exponentially inactivated at 55°. The reductase activity in extracts from patients *LM* and *BM* (the sisters), decreased to 22% and 38%, respectively, of the initial values. In repeated experiments the heat inactivation of reductase activity in extracts of cells from *LM* and *BM* closely resembled the normal control subjects in total extent and time course of inactivation. The reductase activity in extracts from *WMa*, a fourth, unrelated patient, was also completely inactivated but somewhat less rapidly than with patient *CP*. The same pattern of inactivation was obtained with extracts that had been freed initially of low molecular weight substances by gel filtration (Sephadex G-25 in 30 mM K phosphate, pH 6.3).

Fig. 2. Thermal stability of methylenetetrahydrofolate reductase activity. Extracts of fibroblasts containing 0.3–0.6 mg protein were added to a solution without FAD (A) or with 72 μ M FAD (B) containing all other components of the standard reaction mixture except substrate. After incubation at 55° for the time indicated, the reaction tubes were returned to the ice bath, the $[5-^{14}C]$ methylenetetrahydrofolate substrate was added and, after incubation at 37° for 60 min, the reactions were terminated and the product measured as described previously (7). Activity is expressed as counts per min of formaldehyde formed per reaction mixture.

In additional experiments (data not shown), when 72 μ M FAD was added to the reaction mixtures after the 55° preincubation but prior to assay, the reductase activities observed were unchanged, indicating that the FAD cofactor is not able to reactivate the reductase.

DISCUSSION

The specific activities of methylene- H_4 PteGlu reductase in extracts of confluent fibroblasts from patients *CP*, *LM*, and *BM* were well below normal when assayed under standard conditions as has been reported previously by Mudd *et al.* (6), Shih *et al.* (9), and Freeman *et al.* (3). The expression of the deficiency in fibroblasts from these patients and the fact that methylene- H_4 PteGlu reductase activity is easily detected in normal amniotic fluid cells (7) raise the possibility that this disorder can be diagnosed prenatally. Fibroblasts from the parents of *CP* yielded reductase activities intermediate between those of *CP* and the normal control subjects, suggesting autosomal recessive inheritance of this inborn error of folate metabolism. We have no explanation for the fact that these presumably heterozygous values were reproducibly less than half of the normal mean. Although in the present studies the culture conditions used were those that yield reproducible values, it is important to note that the reductase activity normally fluctuates by 2.5-fold or more depending on the stage of cell growth, being lowest in the logarithmic phase and highest deep in confluency (7). If uncontrolled, these growth-related fluctuations could produce an erroneous diagnosis of heterozygous reductase deficiency.

Although the omission of FAD from the standard assay reaction mixture at 37° did not affect the reductase specific activity in extracts of normal fibroblasts (6, 7), the reductase activity was much less stable at elevated temperatures in the absence of added FAD than when 72 μ M FAD were added (Fig. 2). This presumably represents cofactor stabilization through formation of an enzyme-FAD complex, as originally postulated by Donaldson and Keresztesy (1).

Although the pH optimum and K_m for 5-methyl- H_4 PteGlu of the residual methylene- H_4 PteGlu reductase activity in the two patients tested were normal, the thermostabilities differed greatly. When incubated at 55° in the presence of 72 μ M FAD (Fig. 2), two slopes of inactivation were apparent in extracts from two unrelated normal control subjects and inactivation was only partial in the 30-min test period. Although the specific reductase activities were reduced in all patients, the residual reductase activity in patients *LM* and *BM* was similar to normal in regard to both the degree of inactivation and the biphasic time course. The reductase in patient *CP* was least thermostable and was inactivated with a single exponential slope, whereas the reductase in *WMA* was inactivated at an intermediate biphasic rate. Thus the two normal subjects and the two sisters, *LM* and *BM*, show consistent patterns of inactivation in the presence of added FAD, whereas the two other unrelated patients, *CP* and *WMA*, are distinctly different. These results provide strong evidence that the reductase deficiency in these three families is due to different alleles. In all three families the mutations have decreased the catalytic capacity and/or the total amount of methylene- H_4 PteGlu reductase. However, in *CP* and, to a lesser extent, in *WMA*, the interaction with FAD has also been affected. No evidence has been published to indicate whether mammalian methylene- H_4 PteGlu is composed of subunits. Purification of the normal and mutant reductase proteins would probably be a prerequisite to more detailed studies on the molecular basis of reductase deficiency. Furthermore, too little

information is presently available regarding the relevant conditions *in vivo* to know what role this altered thermostability might play in the clinical and metabolic manifestations of the methylene- H_4 PteGlu reductase deficiency in these patients.

Mudd *et al.* (6) first suggested that the specific genetic lesion in *CP* might be different from that in the sisters and raised the possibility that the reductase activity in *CP* might be unstable when assayed without FAD, although the possibility of a primary abnormality in flavin metabolism was not ruled out. The data presented here strongly suggest that in *CP* and *WMA* there is a mutationally induced structural defect in the aporeductase as the basis for the observed alteration in thermostability, presumably reflecting reduced ability to bind the FAD cofactor. Thus the methylene- H_4 PteGlu reductase deficiency, detected to date in three unrelated families, results from at least three distinct mutant alleles.

CONCLUSION

Methylene- H_4 PteGlu reductase activity in crude extracts of four patients in three families with a deficiency of this enzyme ranged from 14–20% of normal. The activities in extracts from the parents of one patient were 40% and 35% of normal, suggesting that this inborn error of metabolism is inherited in an autosomal recessive pattern. Thermal stability studies demonstrated a different pattern of heat inactivation in each of the families, which suggests that the reductase deficiency in these unrelated families results from at least three distinct mutant alleles.

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