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On the Mechanism of Pyridoxine Responsive Homocystinuria. II. Properties of Normal and Mutant Cystathionine β-Synthase from Cultured Fibroblasts*

(apoenzyme–coenzyme interaction/thermostability)

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ABSTRACT **Cystathionine β-synthase [L-serine hydro-lyase (adding homocysteine), EC 4.2.1.22] was studied in cultured skin fibroblasts from two control subjects and three patients with pyridoxine-responsive homocystinuria. In crude cell sonicates, cystathionine synthase activity detected in each mutant line was less than 5% of control values. After differential centrifugation, ammonium sulfate fractionation, and calcium phosphate gel treatment, the specific activity of synthase from control lines increased 5- to 7-fold with 70–79% yield. These same steps led to only 2- to 3-fold purification of mutant synthase and a reduced yield (26–44%). Michaelis–Menten analyses with the partially purified enzyme revealed that each mutant synthase had a marked reduction in affinity for its coenzyme, pyridoxal 5′-phosphate, as well as reduced affinity and maximum velocity for both co-substrates, L-homocysteine and L-serine. Even at saturating concentrations of coenzyme, mutant synthase activity was less than 3% of control. Mutant synthase was also far more thermolabile than control enzyme. In the absence of added coenzyme, heating for 10 min at 55° led to complete loss of mutant activity whereas control activity was reduced by 60%. Significantly, addition of saturating concentration of coenzyme prior to heating increased thermostability of both control and mutant synthase, the fractional increase being considerably greater in the mutants. We conclude that these patients suffer from a mutation of the synthase apoenzyme which impairs coenzyme binding, and that this primary abnormality results in reduced total enzyme activity in two ways: by reducing holoenzyme formation; and by accelerating apoenzyme degradation. We propose that pharmacologic amounts of pyridoxine increase holoenzyme formation modestly, thereby enhancing catalytic activity and slowing apoenzyme turnover.**

In 1962, the disorder "homocystinuria" was discovered in children with dislocated optic lenses, osteoporosis, skeletal abnormalities, and mental retardation whose plasma and urinary concentrations of methionine and homocystine were markedly increased (1, 2). This form of inherited homocystinuria was shown to result from a striking reduction in activity of the hepatic enzyme, cystathionine β-synthase [L-serine hydro-lyase (adding homocysteine), EC 4.2.1.22], which requires pyridoxal 5′-phosphate (pyridoxal-*P*) as a cofactor, and catalyzes the condensation of homocysteine and serine to form cystathionine (3, 4). The enzyme deficiency was demonstrated subsequently in brain, cultured skin fibroblasts, and phytohemagglutinin-stimulated lymphocytes of such homocystinuric patients (5).

Barber and Spaeth (6) first reported patients with cystathionine β-synthase deficiency who responded to pharmacologic doses of pyridoxine, the vitamin precursor of pyridoxal-*P*, with complete return of their plasma and urinary methionine and homocystine concentrations to normal. This observation was confirmed subsequently by several groups of investigators (7) in some, but not all such patients. The biochemical basis for this pyridoxine responsiveness has not been defined adequately. In some responsive patients, the synthase activity in crude liver homogenates or fibroblast sonicates has been increased slightly (8, 9) or significantly (10) by pyridoxal-*P* supplements, whereas in other responsive patients no such enhanced activity has been observed (9, 11, 12). Uhlendorf *et al.* (13) argued that the only biochemical characteristic that discriminates between pyridoxine-responsive and -unresponsive patients was the level of residual, basal synthase activity: responsive patients retain a small fraction of activity; unresponsive patients have no demonstrable activity. In this study, the mechanism of pyridoxine responsiveness is characterized further by studying the properties of cystathionine β-synthase from cultured fibroblasts in control and pyridoxine-responsive patients.

MATERIALS AND METHODS

Materials. L-[3-^{14}C]serine (specific activity, 48–56.5 mCi/mmol) was obtained from Amersham-Searle. Before use, it was further purified by ion exchange resin column chromatography (14). Unlabeled L-cystathionine and L-homocysteine thiolactone were purchased from California Biochemical Co. (Calbiochem). L-serine and ammonium sulfate (ultra pure special enzyme grade) were obtained from Schwarz-Mann Research Laboratories, and pyridoxal-*P* and Tris hydroxyaminomethane from Sigma. Dowex AG 50W-X4 H^+ (200–400 mesh) was purchased from BioRad Laboratories.

Cultured Cells. Control cells were propagated from skin biopsies obtained from two healthy, young adult males. Mutant cell lines were established from three patients with documented pyridoxine-responsive cystathionine β-synthase deficiency. Clinical and metabolic findings in the first two patients, J.K. and E.Y. (hereafter designated mutant 1 and mutant 2, respectively), have been described previously (9).

Abbreviation: pyridoxal-*P*, pyridoxal 5′-phosphate.

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TABLE 1. *Partial purification of cystathionine β-synthase from cultured fibroblasts obtained from controls and from patients with pyridoxine-responsive homocystinuria*

	Controls		Mutants		
Parameter	1§	2	1§	2¶	3
Prep. I					
S.A.*	11.9	6.2	0.36	0.21	0.08
R.P.†	1	1	1	1	1
Yield‡	100	100	100	100	100
Prep. II					
S.A.	17.8	10.1	0.43	0.36	0.09
R.P.	1.5	1.7	1.2	1.7	1.2
Yield	92	87	66	82	71
Prep. III					
S.A.	42.3	34.2	0.67	0.47	0.15
R.P.	3.6	5.6	1.9	2.2	1.9
Yield	88	83	40	38	52
Prep. IV					
S.A.	55.3	45.3	0.99	0.40	0.20
R.P.	4.6	7.3	2.8	1.9	2.5
Yield	79	70	26	35	44

* Enzyme specific activity (S.A.) is expressed in units/mg of protein. Units are defined as the number of nmol of cystathione formed per mg of protein per 60 min at 37°. Synthase was assayed as described in *Materials and Methods*, with 7.5 to 9.0 × 10^5 dpm of L-[3-^{14}C]serine per μmol of total L-serine in control assays and twice this amount of L-[3-^{14}C]serine in mutant assays.

† Relative purity (R.P.) compared to arbitrary value of 1 in Preparation I.

‡ Yield expressed as percent of total activity found in Preparation I.

§ Values are the mean of separate observations on two different batches of pooled cells.

¶ Values are the mean of separate observations on three different batches of pooled cells.

The third patient, designated mutant 3, is a 9-year-old retarded white boy with dislocated optic lenses, markedly elevated plasma methionine and homocystine concentrations (1.72 mg/100 ml and 3.81 mg/100 ml, respectively), undetectable plasma cystine, and increased urinary homocystine. After 2 weeks of administration of pyridoxine (500 mg/day), plasma homocystine and methionine fell to normal.

Skin fibroblast monolayers were grown at 37° in Bellco Roller bottles (1410 cm²) in Eagle's minimal essential medium (MEM from Grand Island Biologicals) containing pyridoxal hydrochloride (1.0 mg/liter), and supplemented with nonessential amino acids, neomycin (100 μg/ml), and 10% fetal calf serum. Each cell line was monitored monthly for mycoplasma infection by means of broth and agar slant techniques and only cells free of mycoplasma were used. Confluent monolayers in their 8th through 25th serial passage were harvested with 0.05% trypsin, washed twice with phosphate-buffered saline (pH 7.4), centrifuged at 600 × *g* for 5 min at 4°, and stored at −70°.

Partial Purification of Cystathionine Synthase. All procedures were carried out at 4°. Frozen fibroblast pellets were thawed, pooled, suspended in 5 volumes (V/V) of pre-cooled 0.1 M potassium phosphate buffer at pH 7.5, and disrupted by sonicating in several 10-sec bursts at setting 3 in a Branson sonifier (Branson Instruments, Co., Stamford, Conn.). This crude cell sonicate (designated as Preparation I in Table 1) was centrifuged at 105,000 × *g* for 60 min in a Spinco model L ultracentrifuge, and the precipitate consisting of cell debris and fragmented membranes was discarded. Saturated ammonium sulfate solution (pH 7.5) was added dropwise, while stirring magnetically, to the clear supernatant (Preparation II) until 40% (V/V) saturation was achieved. The mixture was allowed to stand with stirring for 1 hr. The precipitate was collected by centrifugation at 34,000 × *g* for 30 min (Sorvall RC2B), resuspended in 0.1 M potassium phosphate buffer (pH 7.5), and dialyzed for 15–18 hr against two changes of this buffer. The dialyzed fraction (Preparation III) was treated twice with calcium phosphate gel (1 g of aged gel per 1 g of tissue protein). This mixture was stirred magnetically for 2 hr, and centrifuged at 750 × *g* for 10 min. The supernatant was collected and dialyzed against 0.1 M potassium phosphate buffer (pH 7.5) for 15–18 hr. This preparation (Preparation IV) was used in subsequent biochemical studies. Usually one control and one mutant cell line were purified simultaneously.

Enzyme Assay. Cystathionine β-synthase activity was assayed by modification of the method of Mudd *et al.* (14). Enzyme reaction mixture (0.4 ml) containing 5 μmol of L-homocysteine, 1 μmol of L-serine, 0.07 μmol of L-cystathionine, 0.4 μmol of pyridoxal-*P*, 60 μmol of Tris·HCl buffer (pH 8.3), 0.3–0.4 μCi of L-[3-^{14}C]serine, and the enzyme preparation (0.05–0.5 mg of protein) was incubated for 60 min at 37°. The [^{14}C]cystathionine formed was eluted in 8–10 ml of 3 N ammonium hydroxide (14). The entire eluate was dried, the residue dissolved in water, and counted in Aquasol in a Packard liquid scintillation spectrometer. Protein concentration was determined by the method of Lowry *et al.* (15). We define 1 unit of enzyme activity as that which leads to the formation of 1 nmol of L-cystathionine per hr at 37°. Enzyme specific activity is expressed as units/mg of tissue protein.

Heat Activation. Thermolability of the synthase was determined by heating samples of enzyme Preparation IV for 10 min at various temperatures or for various periods of incubation at a constant temperature (55°) in a metabolic shaker. The heated samples were then thrust into an ice bath, cooled for 15–30 min, and assayed for enzymatic activity. Control and mutant enzymes were always studied simultaneously.

Calculations. In the Michaelis–Menten analyses, the double reciprocal method of Lineweaver and Burk (16) was used to estimate K_m and V_{max}. Statistical analyses were conducted with Student's *t* test.

RESULTS

Pooling and Storage of Intact Fibroblasts and Cell Extracts. Each roller bottle yielded 100–210 mg wet weight of cells. Purification was carried out on pooled cells ranging in wet weight from 1700 to 3600 mg (8 to 20 roller bottles). Specific activity of the synthase in each control and mutant line was virtually identical in sonicates assayed on the day of harvest and in sonicates prepared from cell pellets frozen at −70° for up to 3 months. Loss of control synthase activity was negligible in Preparation IV kept at −70° for 30 days. Mutant cell extracts were never stored for more than 10 days and no loss of activity was observed during this interval.

Assay of Cystathionine β-Synthase Activity. Preparation IV from control 1 was used to standardize the conditions for

optimal assay. As noted in Fig. 1A and B, [^{14}C]cystathionine formation was linear with protein content up to 0.5 mg and with time up to 60 min. In Tris·HCl buffers ranging from pH 6 to pH 10.9, a pH optimum of 8.3 was found (Fig. 1C). All subsequent assays were carried out for 60 min at pH 8.3. The sensitivity of Mudd's original hepatic assay was increased by using L-[^{14}C]serine of higher specific activity and by counting the total dry residue rather than an aliquot thereof. In most studies with extracts from mutant lines, twice as much [^{14}C] serine was added to the reaction mixture to increase the amount of labeled product.

Purification of Cystathionine β-Synthase. The results of enzyme purification studies in two control and three mutant lines are shown in Table 1. Specific activity of enzyme in the crude sonicate (Preparation I) was 11.9 and 6.2 units in controls 1 and 2, respectively, and increased stepwise to 55.2 units in Preparation IV from control 1 and to 45.3 units in control 2, a 4.6- to 7.3-fold purification. Overall enzyme yield was 79 and 70% in the two control lines. Such findings were observed consistently in five different batches of cells from control 1. The three mutant lines behaved differently from control lines during these purification steps. Specific activity in crude sonicates was less than 5% of that in the controls, but was detectable in each mutant line. The activity increased only 2- to 3-fold during purification and overall yields ranged from 26 to 44%.

Michaelis–Menten Analyses. Kinetic analyses for both substrates, L-homocysteine and L-serine, were carried out with Preparation IV from control 1 and mutant 1. Pyridoxal-*P* concentration was held constant at 1.0 mM. The *control* enzyme demonstrated convergent, linear kinetics at three or four different concentrations of each co-substrate yielding a common, abscissa intercept. For L-homocysteine the apparent K_m was 16.1 mM (Fig. 2A), and the V_{max} was 89 units and 212 units, respectively, at L-serine concentrations of 2.5 and 10 mM. The apparent K_m for L-serine was estimated at 1.4 mM and the V_{max} (at 10 mM L-homocysteine concentrations) at 35.5 units. Linear kinetics were also observed in mutant 1 (Fig. 2B). The estimated K_m's for L-homocysteine (31.3 mM) and L-serine (4.6 mM) were two to three times those found in the control, whereas the V_{max}'s for each substrate were reduced by a factor of 10 or more at comparable concentrations of the co-substrate, 9 units for L-homocysteine at 2.5 mM L-serine, 3.6 units for L-serine at 10 mM L-homocysteine. Such kinetic analyses were not carried out in the other lines due to limited availability of cells.

Effect of Addition of Pyridoxal-P In Vitro. Cystathionine β-synthase activity in Preparation IV from the two control and three mutant lines was determined in the absence of added pyridoxal-*P* and in the presence of graded concentrations of cofactor. In both control lines, specific activity increased steeply when pyridoxal-*P* was increased from 0 to 0.1 mM, and plateaued by 1 mM (Fig. 3A), yielding an estimated K_m for pyridoxal-*P* of 0.01–0.02 mM under standard conditions for both co-substrates (L-homocysteine 12.5 mM; L-serine 2.5 mM). Addition of pyridoxal-*P* also increased the enzyme activity 2- to 4-fold in each mutant line (Fig. 3A and B). However, maximal synthase activity in mutants 1, 2, and 3, respectively, was only 2.7, 0.5, and 0.4% of that in controls—even when pyridoxal-*P* was added at concentrations approaching its solubility limit. Furthermore, the slope of incremental synthase activity with increasing concentrations of pyridoxal-*P* was much more shallow in the mutant than in the control lines, indicating that the affinity for coenzyme was less than 5% of that observed in either control preparation.

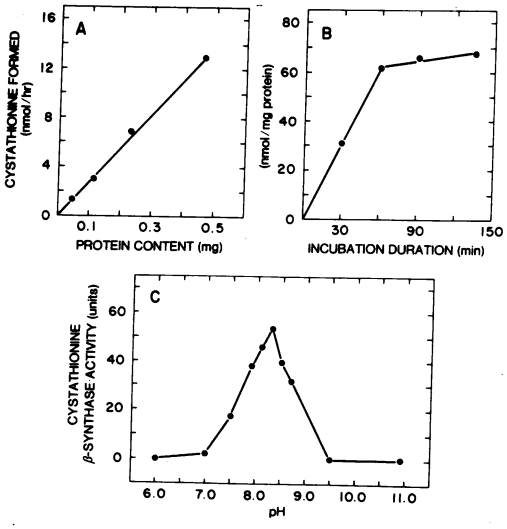

FIG. 1. Assay conditions for cystathionine β-synthase activity in enzyme Preparation IV from cultured skin fibroblasts of control 1. (A) Relationship between cystathionine formation and protein content of the assay mix at pH 8.3, (B) Relationship between cystathionine formation and duration of assay at pH 8.3, (C) Estimation of pH optimum using Tris·HCl buffers. Each point represents the mean of duplicate observations in a representative experiment.

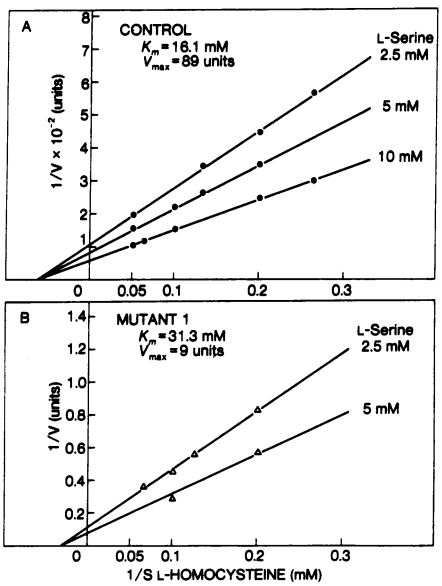

FIG. 2. Kinetic analysis for L-homocysteine in control 1 (*upper panel*) and mutant 1 (*lower panel*) enzyme at different concentrations of the co-substrate, L-serine. Enzyme Preparation IV was used throughout. The apparent K_m's were calculated from the common abscissa intercept of the converging double reciprocal plots. The V_{max}'s were estimated at an L-serine concentration of 2.5 mM. Pyridoxal-*P* concentration was 1.0 mM in all experiments. Note the 20-fold difference in ordinate scales between the upper and lower frames.

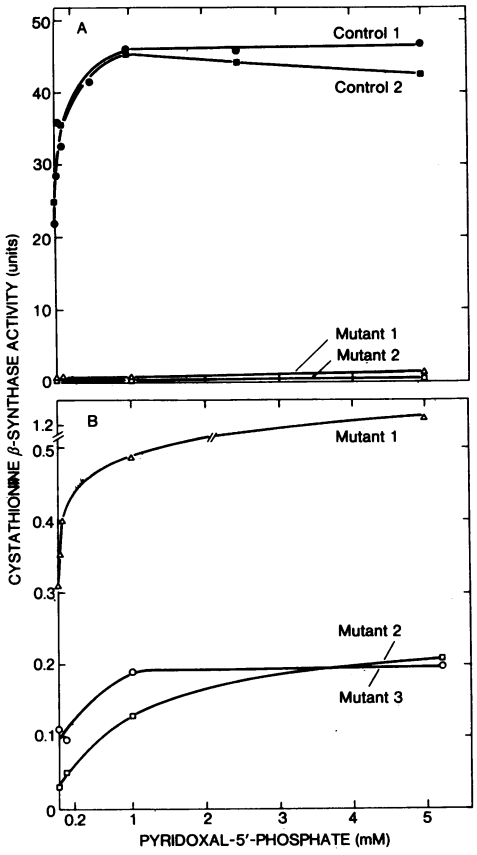

FIG. 3. Effect of increasing concentrations of pyridoxal-*P* *in vitro* on cystathionine β-synthase activity in enzyme Preparation IV from control and mutant fibroblasts (*upper panel*). Co-substrate concentrations were 2.5 mM for L-serine and 12.5 mM for L-homocysteine. Each point represents the mean of duplicate observations in a representative experiment in which enzyme from a control and a mutant line were assayed simultaneously. In the *lower frame*, the data for the three mutant lines are depicted on a 100-fold expanded scale.

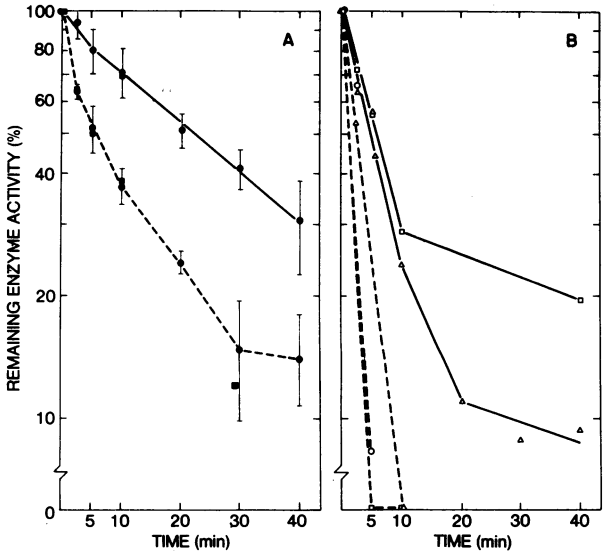

FIG. 4. Heat inactivation of cystathionine β-synthase activity in enzyme Preparation IV from control (*left*) and mutant (*right*) fibroblasts. Aliquots of each preparation were incubated at 55° for the duration noted on the abscissa and were then chilled to 4° for 15–30 min prior to synthase assay. Remaining synthase activity of the heated samples (ordinate) is expressed as the percent of synthase activity determined in paired samples not subjected to 55° incubation. In both frames, data joined by broken lines were obtained when no pyridoxal-*P* was added during the 55° incubation whereas in those joined by solid lines, 1.0 mM of the cofactor was added during heating. The pyridoxal-*P* concentration of the assay mix was 1.0 mM throughout. In the *left frame*, the data for control 1 (●) reflect the mean ± one standard deviation of five separate experiments in the presence of cofactor and three experiments in the absence of cofactor. Statistically significant differences ($P < 0.05$) were observed at all time points when the results obtained in the absence of cofactor were compared with those in the presence of cofactor. Confirmatory single experiments were carried out in control 2 (■). In the *right frame*, the data for mutant 1 (△) are the mean of two separate experiments whereas those for mutant 2 (□) and mutant 3 (○) are from single studies.

Synthase activity from mutant 1 was greater than that in mutants 2 or 3, in the presence or absence of added pyridoxal-*P* (Fig. 3B). This is of interest because mutant 1 also responds to the smallest dose of pyridoxine *in vivo* (9). Furthermore, it should be pointed out that, whereas synthase activity in a crude sonicate from mutant 2 failed to increase at any added concentration of pyridoxal-*P* (9), his partially purified enzyme did show demonstrable stimulation by cofactor.

Thermolability of Control and Mutant Cystathionine β-Synthase. Heating Preparation IV from control 1 to temperatures above 50° for 10 min led to a progressive fall in specific activity. Detailed thermostability studies were then carried out by heating the partially purified enzyme from each subject to 55° for various intervals. Pyridoxal-*P* was added to the assay mix at a concentration of 1 mM. As shown in Fig. 4A, addition of this same concentration of pyridoxal-*P* before heating stabilized control synthase significantly. Thus, heating control 1 enzyme for 10 min in the absence of pyridoxal-*P* led to a 63% fall in activity compared to only a 30% fall when pyridoxal-*P* was present. The three mutant enzymes were far more thermolabile than either of the controls (Fig. 4B). In the absence of pyridoxal-*P*, virtually all activity disappeared in mutants 2 and 3 after 5 min of heating 2, and in mutant 1, after 10 min. In the presence of cofactor, however, the mutant enzymes were significantly more stable, albeit still decaying at a rate considerably faster than that observed in the controls.

DISCUSSION

Cystathionine β-synthase has been purified extensively from rat liver by several groups of investigators (4, 17–19), but no previous attempts at purification of the human enzyme have been reported. We patterned our purification procedures after those used in rat liver and observed that the human enzyme has a pH optimum and apparent K_m's for L-serine and L-homocysteine similar to those of the rat enzyme.

When identical steps of purification were carried out on two control and three mutant lines, a number of differences were observed. First, the specific activity of the synthase in the crude sonicates from mutant lines, although detectable, was less than 5% of that noted in the controls. Second, only a 2.2- to 2.8-fold increase was noted in the mutants, whereas a 4.6- to 7.3-fold increase in specific activity was achieved during purification in the control lines. Third, enzyme yield after

partial purification was 26–44% in the mutants compared to 70–79% in the control cells. These findings suggest that either the chemical properties of the mutant enzyme differ enough from those of the control so that response to such reagents as ammonium sulfate and calcium phosphate gel is altered or that the mutant enzyme is more readily denatured by the chemical treatments employed. It should be pointed out that pyridoxal phosphate was not added during any of the purification steps. It seems likely that addition of cofactor would enhance purification and improve the yield, particularly in the mutants.

Examination of some chemical and kinetic properties of the partially purified synthase from control and mutant lines also revealed some impressive differences. Whereas the affinity of mutant enzyme for L-serine and L-homocysteine was decreased 2- to 3-fold, affinity for pyridoxal phosphate was 20-fold less than that of control enzyme. Even with saturating quantities of pyridoxal-*P*, however, the specific activity in the mutants was less than 3% of that in the control. Mutant enzyme was also much more thermolabile than control. This difference was most dramatic when pyridoxal-*P* was not present during heating, but was also obvious when cofactor was added. Thermostability of both control and mutant synthase was enhanced considerably by the addition of pyridoxal-*P* during heating.

Mudd *et al.* (10) conducted similar thermostability studies in trypsin-treated crude liver extracts from two controls and two pyridoxine-responsive patients with cystathionine β-synthase deficiency. They too found that addition of pyridoxal-*P* during heating (59°) considerably increased the thermostability of synthase activity in controls and patients, and that under these conditions, synthase activity from the patients' livers decayed more rapidly than that obtained from controls. However, no difference in synthase thermostability was observed between patient and control liver when pyridoxal-*P* was omitted during the heating step. The latter observation is not in agreement with our own and is unexplained.

Our results, taken in conjunction with those of Mudd, Uhlendorf, and their colleagues (10, 13), lead us to propose a biochemical interpretation of pyridoxine-responsive cystathionine β-synthase deficiency. Basic to this construct is the notion that a structural mutation of the synthase apoenzyme results in marked impairment of apoenzyme–coenzyme interaction. Because of this structural change, the catalytic activity of the mutant synthase is much reduced for two reasons: a reduced rate of formation of active holoenzyme; and an increased rate of degradation of the mutant apoenzyme. Administration of pharmacologic doses of pyridoxine leads to increased cellular concentrations of pyridoxal-*P* which, in turn, enhance holoenzyme activity modestly by increasing the rate of holoenzyme formation and by decreasing the rate of apoenzyme degradation. The following observations on other pyridoxal-*P* dependent enzymes in animal cells are consistent with this rationale: the reduced hepatic apoenzyme content of several such enzymes in pyridoxine-deficient rats (20, 21); the impressive, positive correlation between the relative rates of coenzyme dissociation and degradative half life of several pyridoxal-*P*-requiring, hepatic enzymes (22); and the increased thermostability of tyrosine aminotransferase *in vitro* in the presence of pyridoxal-*P* (23, 24). We are aware that *in vitro* thermostability need not reflect accurately *in vivo* enzyme turnover, and that thermostability *in vitro* may be influenced by the presence of other large and small molecules in impure enzyme preparations. Nonetheless, the biochemical explanation proposed herein is consistent with both the published data for cystathione β-synthase deficiency and with that relating to other mutant human enzymes (25). Definitive evidence for such a biochemical mechanism will depend on the availability of highly purified normal and mutant human cystathione β-synthase and on measurements of apoenzyme and holoenzyme content as well as catalytic activity.

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