Homocystinuria

EVIDENCE FOR THREE DISTINCT CLASSES OF CYSTATHIONINE β-SYNTHASE MUTANTS IN CULTURED FIBROBLASTS

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ABSTRACT We have compared in vivo pyridoxine responsiveness with in vitro cystathionine β-synthase activity in extracts of confluent fibroblasts from 14 synthase-deficient patients. Enzyme activity was measured with and without addition of its cofactor, pyridoxal-5′-phosphate, using a radioisotopic assay which detects as little as 0.25% of control activity. Six of seven lines from responsive patients had measurable activity without the added cofactor (0.6–15% of mean control). Two of these lines showed a five- and sevenfold stimulation of cystathionine β-synthase activity with added pyridoxal-5′-phosphate; in the other four, the cofactor addition increased activity only modestly, as in controls. Two of seven lines from nonresponsive patients had measurable activity (each 3% of mean control) which increased two- and fivefold with the added cofactor. Cystathionine β-synthase activity was undetectable in one line from a responsive patient and in five lines from nonresponsive ones. To characterize control and mutant synthase further, dissociation constants for pyridoxal-5′-phosphate were estimated and thermostability (54°C) was studied in two control and five mutant lines. In one mutant, both parameters were normal; in the others, the affinity for the cofactor was reduced 3- to 11-fold and thermostability was much impaired. We conclude that at least three general classes of cystathionine β-synthase mutants exist: those with no residual activity; those with reduced activity and normal affinity for pyridoxal-5′phosphate; and those with reduced activity and a reduced affinity for the cofactor. Pyridoxine responsiveness in vivo cannot be correlated simply with the presence or absence of residual synthase activity in vitro or with stimulation of in vitro enzyme activity by cofactor.

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INTRODUCTION

The most common type of inherited homocystinuria is characterized clinically by dislocated lenses, skeletal abnormalities, intravascular thromboses, and mental retardation, and chemically by increased concentrations of homocystine and methionine in plasma and urine (1, 2). A marked deficiency of cystathionine β-synthase (CS)[1] activity has been demonstrated in the liver (3), brain (4), cultured skin fibroblasts (5) and phytohemagglutinin-stimulated lymphocytes (6) of affected patients. Cystathionine β-synthase (L-serine hydro-lyase [adding homocysteine], EC 4.2.1.22) requires pyridoxal-5′-phosphate (PLP) as a cofactor (7–9) and catalyzes the condensation of serine and homocysteine to form cystathionine.

Approximately half of the reported patients with CS deficiency show a return of plasma and urine methionine and homocystine concentrations to normal or near normal values in response to treatment with pharmacologic amounts of pyridoxine, the vitamin precursor of PLP (10, 11). Several investigators have attempted to define the biochemical mechanism of this response. In some responsive patients, the addition of PLP to crude liver and cultured fibroblast homogenates was shown to result in either slight or significant increases in the activity of CS, whereas in other responsive patients, no stimulation of residual activity was observed (12–14). Uhlendorf et al. (15) studied cultured fibroblasts from a large series of in vivo pyridoxine-responsive and nonresponsive patients. Cell lines from 24 of 25 responsive patients exhibited measurable residual CS activity whereas those from 9 of 10 nonresponsive

[1] *Abbreviations used in this paper*: CS, cystathionine β-synthase; PLP, pyridoxal-5′-phosphate.

patients had no detectable activity. They concluded that, in general, in vivo pyridoxine responsiveness is associated with the retention of small amounts of residual CS activity which is enhanced to the same degree as control enzyme by in vitro addition of PLP. Kim and Rosenberg (16) reached different conclusions from studies of PLP kinetics and thermostability on partially purified CS from three other pyridoxine-responsive patients. They found that the mutant enzymes exhibited a reduced affinity for PLP and a decreased thermostability. They proposed that, in the presence of physiologic amounts of PLP, little holo-CS exists and apo-CS turnover is accelerated; and that, when tissue PLP content rises in response to pyridoxine supplements, holo-CS content is increased and apo-CS turnover is decreased. As a result, catalytic activity is enhanced.

In the present study, CS activity was measured in cultured fibroblast extracts from 14 homocystinuric patients with defined responses to pyridoxine treatment. The results suggest an initial classification of CS mutants based on residual activity, affinity of aposynthase for cofactor, and thermostability. The extent to which in vivo pyridoxine responsiveness can be correlated with these in vitro properties of CS is reexamined.

METHODS

Materials. L-[U-^{14}C]Serine was obtained from Schwarz/Mann Div., Orangeburg, N. Y. and from New England Nuclear, Boston, Mass.; PLP and dithiothreitol from Sigma Chemical Co., St. Louis, Mo.; L-cystathionine and L-homocysteine thiolactone from Calbiochem, La Jolla, Calif.; L-serine from Schwarz/Mann; and hydroxylamine hydrochloride from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Patients. Skin biopsies for culture from 14 patients were obtained after informed consent had been granted. These patients were diagnosed as having homocystinuria due to CS deficiency. The diagnosis was made on the basis of clinical findings and(or) concentrations of sulfur amino acids in plasma and urine (Table I). The effect of pharmacologic doses of pyridoxine (50–1,000 mg/day) on plasma and urine sulfur amino acid concentrations had been defined in each of these patients, who were then classified as being completely, partially, or nonresponsive to pyridoxine.

Cultured cells. Skin fibroblasts obtained from pinch or punch biopsies were cultured in 32-ounce or Bellco roller bottles (Bellco Glass, Inc., Vineland, N. J.) at 37°C in a 5% CO_2/95% air atmosphere using Eagle's minimum essential medium containing pyridoxal hydrochloride (1 mg/liter) and kanamycin (100 μg/ml), supplemented with 1% nonessential amino acids and 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). Cells were grown to confluence (from 4 to 7 days after subculture), then harvested with 0.1% trypsin solution, washed twice with phosphate-buffered saline (pH 7.4), centrifuged at 1,000 *g* for 5 min at 4°C, and then stored as cell pellets at −70°C. All cell lines had been in culture from 8 to 25 serial passages. Cell extracts were prepared by sonication in either 0.05 M Tris/HCl buffer, pH 8.6, or in 0.05 M potassium phosphate buffer, pH 7.5. The supernate obtained after centrifugation at 10,000 *g* for 10 min at 4°C was mixed gently and used for assay.

Enzyme assay. CS activity was assayed by the radioisotopic method of Mudd et al. (4) with the following modifications. The commercial L-[U-^{14}C]Serine was purified before use by a paper chromatographic procedure to be subsequently described for separation of cystathionine from serine. The incubation mix contained, in a total volume of 0.2 ml: L-serine

TABLE I
Clinical Features of CS Deficient Patients

Number	Sex	Age	Major clinical findings	Response to pyridoxine*
		yr		
1	M	22‡	Dislocated lenses; mild mental retardation	Partial
2	M	24‡	Dislocated lenses; mild mental retardation	Complete
3	M	15‡	Dislocated lenses; mental retardation	Complete
4	F	13	Dislocated lenses; mild mental retardation	Complete
5	M	2	None; low methionine diet and pyridoxine supplement from birth	Partial
6	M	11	Dislocated lenses	Partial
7	F	3	Dislocated lenses; mental retardation; one possible thromboembolic episode	Partial
8	M	4	None; low methionine diet from birth	None
9	F	13	Dislocated lenses; mental retardation	None
10	F	1	None; low methionine diet from birth	None
11	M	12	Dislocated lenses; mental retardation	None
12	F	4/12	None; low methionine diet from birth	None
13	M	18	Dislocated lenses; mental retardation; one mild thromboembolic episode	None
14	F	15	Dislocated lenses; mental retardation	None

* Pyridoxine response was defined according to plasma and urine concentrations of methionine and homocystine before and after therapy. "Complete" response refers to return of abnormal values to normal; "partial" response denotes distinct but incomplete correction of amino acid abnormalities; "none" indicates lack of significant change in plasma and urine values after several weeks of supplementation with up to 1,000 mg pyridoxine daily. Patients were not given folate supplements before evaluation of pyridoxine response.
‡ Patients 1, 2, and 3 referred to previously as mutants 2, 1, and 3, respectively (16).

(2.5 mM); 0.8–1.5 μCi [^{14}C]serine; Tris/HCl buffer, pH 8.6 (0.1 M); fibroblast extract containing 200–700 μg of protein; and L-homocysteine (15 mM) prepared freshly from its thiolactone (30.72 mg of the thiolactone was incubated in 0.2 ml of 5 M NaOH at 37°C for 5 min; the pH was adjusted to 8.6 with 2 M HCl and 0.1 ml 1 M Tris/HCl, pH 8.6; then 0.1 ml 0.2 M dithiothreitol and water to a final volume of 1 ml was added). The assay was initiated by addition of the homocysteine solution after a 5-min incubation at 37°C. After a 4-h incubation at 37°C, a 15-μl aliquot of the assay mix was applied to Whatman no. 3 chromatography paper with drying in a hot air stream. Separation of [^{14}C]cystathionine from [^{14}C]serine was achieved by descending chromatography in isopropanol: formic acid:water (80:6:20) with a development time of at least 22 h. The region of the chromatogram corresponding to the position of marker L-cystathionine was cut into strips and the radioactivity determined by immersion in Liquifluor (New England Nuclear)/toluene and counting in a Packard liquid scintillation spectrometer (Packard Instrument Co. Inc., Downers Grove, Ill.). The amount of [^{14}C]cystathionine formed was calculated after subtraction of radioactivity on chromatograms of blank assays containing either no homocysteine or no fibroblast extract. One unit of CS activity is defined as that which produces 1 nmol of L-cystathionine/h. Enzyme specific activity is expressed in units per milligram cell protein determined by the method of Lowry et al. (17).

Resolution of PLP from holo-CS. To prepare apo-CS, extracts were generally treated with hydroxylamine. For the control enzyme, the extract was dialyzed for 24 h at 4° C against two changes of a solution containing 1 mM hydroxylamine in 0.05 M potassium phosphate buffer, pH 7.5, followed by dialysis against two changes of the same buffer without hydroxylamine. In all but one of the five mutant lines tested (no. 4), CS was denatured irreversibly by this treatment. In three of these mutant lines (nos. 2, 5, 14), we used dialysis for 6 and 18 h in hydroxylamine-containing and hydroxylamine-free buffers, respectively. In the fourth (no. 13), simple dialysis in buffer without hydroxylamine for 2 h was employed because other treatments led to irreversible loss of activity.

PLP kinetics. Dissociation constants for PLP were determined by incubation of apoenzyme preparations with varying concentrations of PLP at 37°C for 1 h in 0.05 M potassium phosphate buffer, pH 7.5 before assay of enzyme activity (18).

Thermostability studies. Aliquots of fibroblast extracts in 0.05 M potassium phosphate buffer, pH 7.5, were heated in a shaking water bath at 54°C for varying lengths of time, cooled in ice, and then assayed for CS activity as above. In all experiments, duplicate unheated samples were included. Mutant and control extracts were tested simultaneously.

RESULTS

Standardization of assay. The high sensitivity of our assay for CS activity is indicated in Fig. 1. Serial dilution studies showed that as little as 0.25% of control activity could be detected. Low background levels of radioactivity were obtained under our assay conditions with purified [^{14}C]serine even with direct application of the assay mix onto the chromatogram. This permitted

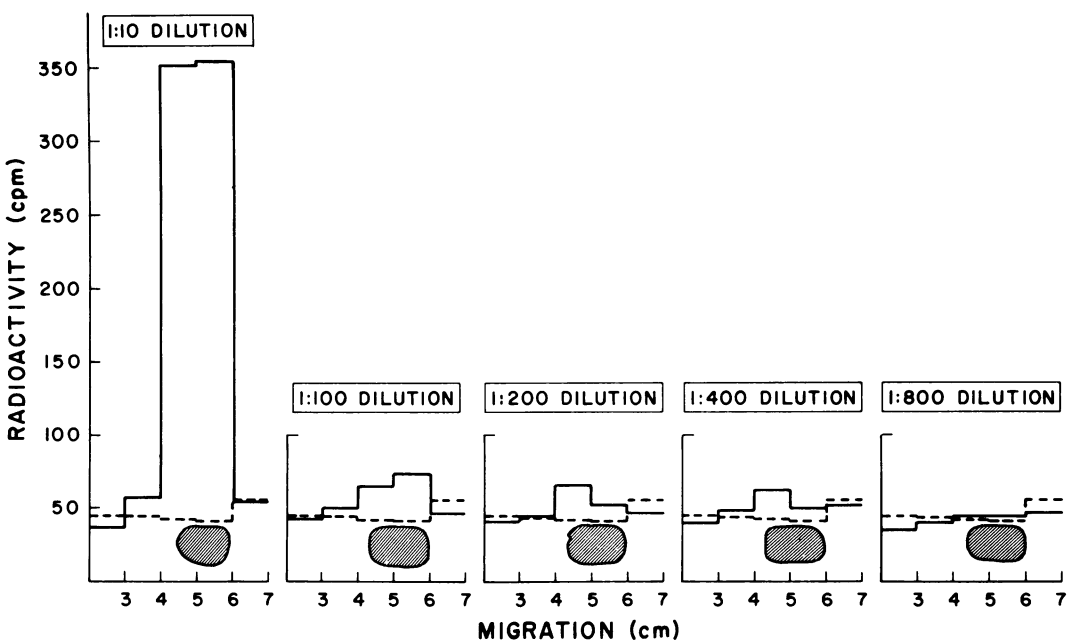

FIGURE 1 Sensitivity of the assay for CS activity. Control fibroblast extract, prepared in 0.05 M Tris/HCl buffer, pH 8.6, was diluted in the same buffer as indicated and assayed in the absence of added PLP. The sample of fibroblast extract diluted 1:10 contained 50 μg protein. The total protein content of each assay was adjusted to 500 μg with bovine serum albumin. After incubation, a 15-μl aliquot of each assay mix was applied to the paper chromatogram. Migration of authentic L-cystathionine is indicated by the cross-hatched area. Radioactivity in this region, after subtraction of blank values (indicated by the broken lines) is taken as a measure of [^{14}C]cystathionine formed in the assay.

omission of an ion-exchange chromatographic step, which is time consuming and results in large losses of [^{14}C]cystathionine (15). In addition, our assay is simpler than that used by Gaull et al. (14) in which cystathionine is measured by automated ion-exchange chromatography. Formation of [^{14}C]cystathionine at 37°C was linear with a 4-h incubation time and with protein content up to 700 μg. There was little variation in CS activity in different batches of cells from the same line and the increase in activity with added PLP was consistent for each individual line. CS activities were not different in the cell extracts prepared by sonication in the Tris/HCl buffer (pH 8.6) or the potassium phosphate buffer (pH 7.5).

CS activity in crude extracts of cultured fibroblasts. CS activity in six control lines (Fig. 2) assayed without added PLP ranged from 2.7 to 7.8 U (mean 5.3) and increased modestly when PLP (1 mM) was added to the assay mix (range 3.4–10.5, mean 6.8 U). CS activities in cell lines from patients with homocystinuria are shown in Fig. 3. In the absence of added PLP, six out of seven lines from in vivo pyridoxine responsive patients and two out of seven lines from in vivo nonresponsive ones have clearly measurable synthase activity (0.6–15 and 3.0–3.3% of mean control values, respectively). CS activity was undetectable in one line from an in vivo pyridoxine-responsive patient and in five lines from nonresponsive ones. Of the lines from responsive patients, two (nos. 2 and 5) showed a large stimulation (five- and sevenfold) of synthase activity when PLP was added in vitro, whereas four showed only modest increases similar to those observed in controls. The lines from nonresponsive patients with detectable synthase activity (nos. 13 and 14) showed two- to fivefold increases in CS activity with added PLP. One exceptional line from an in vivo nonresponsive patient (no. 10) had no activity in the absence of added PLP but 1.1% of control activity when the coenzyme was added.

The in vitro stimulation of CS activity by the addition of PLP may be summarized as follows. There are three types of response: first, a modest increase observed in all the control lines (21–33%) and in four lines from in vivo pyridoxine-responsive patients (25–46%); second, a more striking increase of from two- to sevenfold observed in lines from two in vivo responsive and two

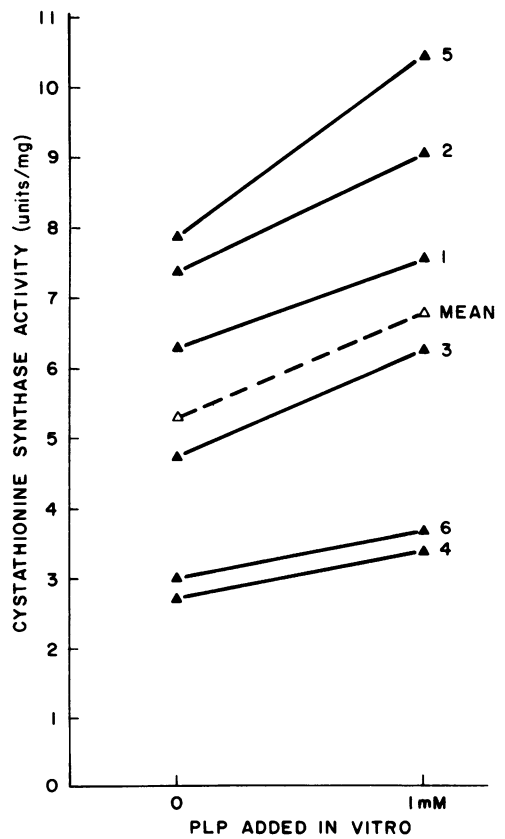

FIGURE 2 CS activity in cultured fibroblast extracts from six controls assayed with and without added PLP. Each value is the mean of determinations on at least two batches of cells. Extracts were prepared by sonication in 0.05 M Tris/HCl buffer, pH 8.6. One unit of synthase activity is defined as 1 nmol cystathionine formed/h. Specific activity is expressed as units per mg cell protein.

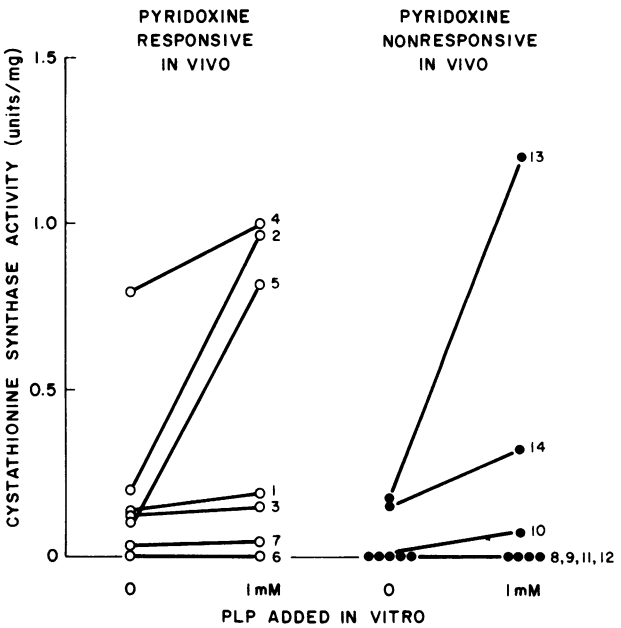

FIGURE 3 CS activity in cultured fibroblast extracts from in vivo pyridoxine-responsive patients (○) and from in vivo pyridoxine nonresponsive patients (●). Extracts were prepared as described in the legend to Fig. 2 and assayed with and without added PLP. Values represent the mean of determinations on at least two batches of cells. Units of activity are as defined in the legend to Fig. 2. Cell line numbers refer to those given in Table I.

in vivo nonresponsive patients; and third, no increase observed in five of the lines with no detectable basal activity.

Effect of storage conditions on CS activity. The above described results were obtained with fibroblast extracts prepared from cell pellets frozen at −70°C. As noted in Fig. 4, however, storage conditions had an appreciable effect on CS activity. Whereas activity was the same in freshly prepared extracts as in extracts from cells frozen at −70°C, storage of extracts at 4°C or dialysis at 4°C against the extraction buffer resulted in marked increases in CS activity (37 and 113% greater than freshly prepared extracts, respectively). Such stimulation was observed in the absence and presence of added PLP, and was noted in all control and mutant extracts exposed to these conditions.

Resolution of control and mutant holo-CS. CS activity was further examined in apo-CS preparations from two control and from the five mutant lines with the greatest residual total enzyme activity. The relative amounts of apo-CS are expressed as a percentage of the total CS of the same preparation assayed with saturating amounts of PLP. Hydroxylamine treatment of extracts from the two controls and mutant 4 resolved nearly all holo-CS to apo-CS, the latter accounting for 93–95% of total CS in the treated extracts compared to 15–35% in the untreated ones. In each of the four other mutant lines studied, apo-CS accounted for a much larger fraction of total CS in the untreated extracts (80% in mutant 2; 76% in mutant 5; 70% in mutant 13; and 50% in mutant 14). The modified resolution conditions employed with these lines (Methods) yielded the following fractional apo-CS values: 98% in mutant 2; 85% in mutant 5; 70% in mutant 13; and 79% in mutant 14.

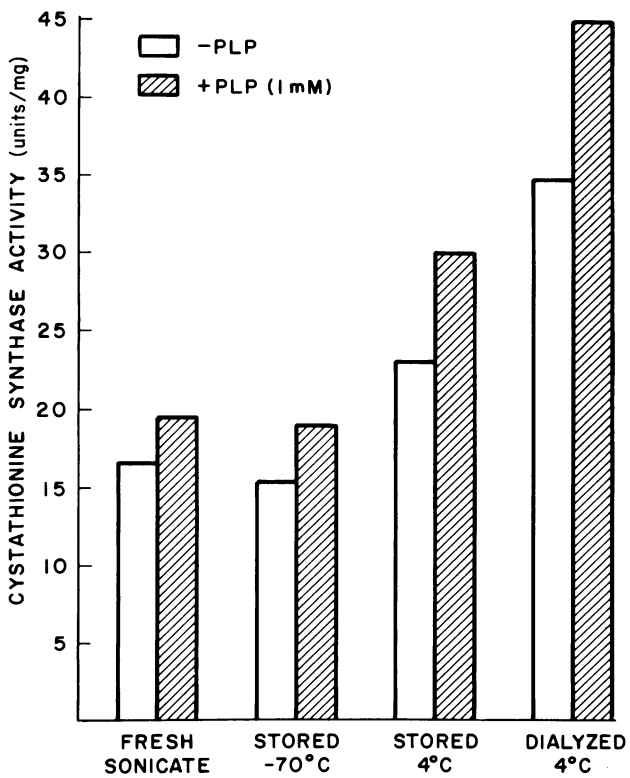

FIGURE 4 Effect of conditions of storage on CS activity in cultured fibroblast extract from control line 5. The extract was prepared by sonication in 0.05 M potassium phosphate buffer, pH 7.5. It was assayed in the absence (open bars) and in the presence (hatched bars) of added PLP (1 mM) under the following conditions: when freshly prepared; after storage at −70°C for 2 days; after storage at 4°C for 2 days; and after dialysis in 0.05 M potassium phosphate buffer, pH 7.5, at 4°C for 2 days. The data presented here are representative of three separate experiments.

Affinity of control and mutant CS for PLP. CS activity in the absence of added PLP and in the presence of increasing amounts of coenzyme was determined using the apo-CS preparations just described. For each preparation tested, typical Michaelis-Menton kinetics were observed as PLP concentrations were increased (Fig. 5). The maximal velocity observed for each mutant line was much less than that in controls, even at saturating concentrations of PLP. The affinity for PLP of CS apoenzyme from mutants 2 and 13 (similar findings were observed for mutants 5 and 14 whose data is not shown) was distinctly reduced when compared to that observed for apoenzyme from controls and mutant 4. Estimated cofactor dissociation constants (K_d PLP) for the two control and five mutant lines tested were calculated from Hill plots (Fig. 6). The K_d PLP for CS from mutant 4 (22 μM) was very similar to that for controls (23 and 30 μM) whereas the K_d PLP for each of the other mutant lines ranged from 2- to 11-fold higher.

Thermostability of control and mutant CS. The data in Fig. 7 show the effect of heat (at 54°C) on total synthase activity in an apoenzyme preparation from control fibroblasts and in its holoenzyme counterpart preparation by addition of PLP (2 mM) before heating. Both preparations showed an initial increase of CS activity during heating, this effect was more prominent for the holoenzyme. Thereafter, there was no appreciable loss of activity over the 45-min interval for the holo-CS whereas the apo-CS showed a steady loss of activity, with only 20% of the initial activity remaining after 45 min. Other experiments (Fig. 8) indicate that mutant apo-CS is more thermolabile than control apo-CS. Once again, thermostability at 54°C was determined in extracts of control and mutant fibroblasts. No attempt was made to resolve PLP from these preparations and PLP was not added before heating. There are clear differences between controls and mutants, the former again showing an initial increase in CS activity followed by a steady loss reminiscent of the plot for apo-CS in Fig. 7. In contrast, mutants 2, 5, 13, and 14 (previously demonstrated to have a much greater frac-

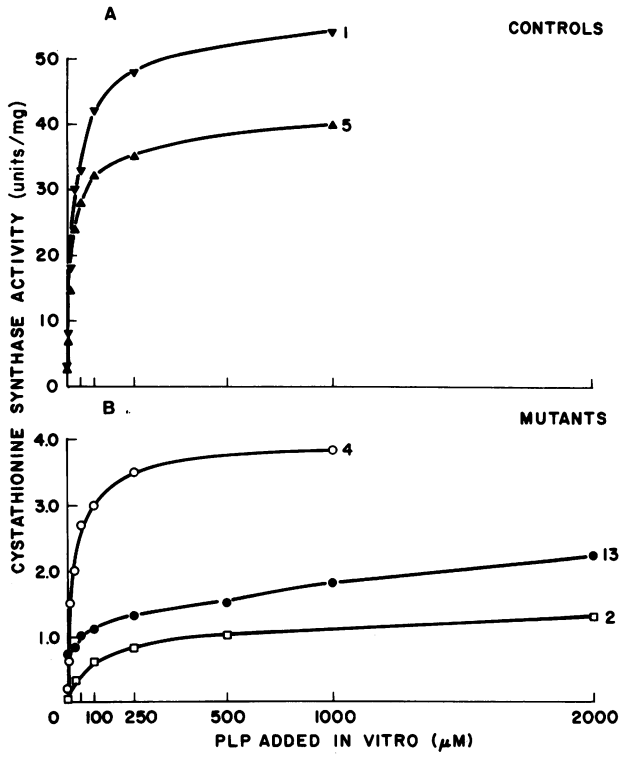

FIGURE 5 The effect of increasing PLP concentration on CS activity in cultured fibroblast extracts from control (A) and mutant lines (B). For controls and mutant 4, CS apoenzyme was prepared by dialysis of the extract in 1 mM hydroxylamine in 0.05 M potassium phosphate buffer, pH 7.5 for 24 h followed by dialysis in the same buffer without hydroxylamine for 24 h. For preparation of apoenzyme from mutant 2, dialysis was used for 6 and 18 h in 1 mM hydroxylamine and buffer, respectively. For mutant 13 the extract was dialyzed in buffer without hydroxylamine for 2 h. Before assay, extracts were preincubated with varying concentrations of PLP for 60 min at 37°C. The scale of the ordinate of B is 1/10th that of A.

tion of their total CS content in the apoenzyme form) showed no initial stimulation and a much more rapid decay of CS activity than did controls. Mutant 4 (whose apo-CS fraction and K_d PLP are similar to controls) showed an initial rise in activity with subsequent decay to intermediate values between those of the controls and other mutants.

DISCUSSION

A sensitive assay for CS activity in extracts of cultured skin fibroblasts from patients with CS deficiency has been used to determine whether in vivo pyridoxine responsiveness can be correlated with in vitro properties of the enzyme. The patients were well characterized, in terms of clinical severity and the effect of pharmacological doses of pyridoxine on plasma and urine concentrations of amino acids. The following findings deserve mention. First, the specific activity of CS in the mutant lines was very reduced when compared to the control values, but varied considerably within the mutant group from nondetectable to 18% of the control values. Second, examination of residual CS activity found in the absence of added PLP confirms (15) a general difference between the in vivo responsive and nonresponsive patients. Cells from all but one of the responsive patients had measurable activity, ranging from 0.6 to 15% of mean control values. In contrast, cells from five of seven nonresponsive patients had no detectable residual synthase activity. However, as in the study of Uhlendorf et al. (15), there are exceptions to the thesis that in vivo pyridoxine responsiveness can be simply correlated with the presence of residual synthase activity in cultured cells. Cells from one responsive patient (mutant 6) had no residual activity even when 0.7 mg of protein was included in the assay, and cells from two nonresponsive patients (mutants 13 and 14) contained measurable activity in all batches of cells studied. Third, four of the mutant cell lines (two from responsive and two from nonresponsive patients) contained synthase activity which showed in-

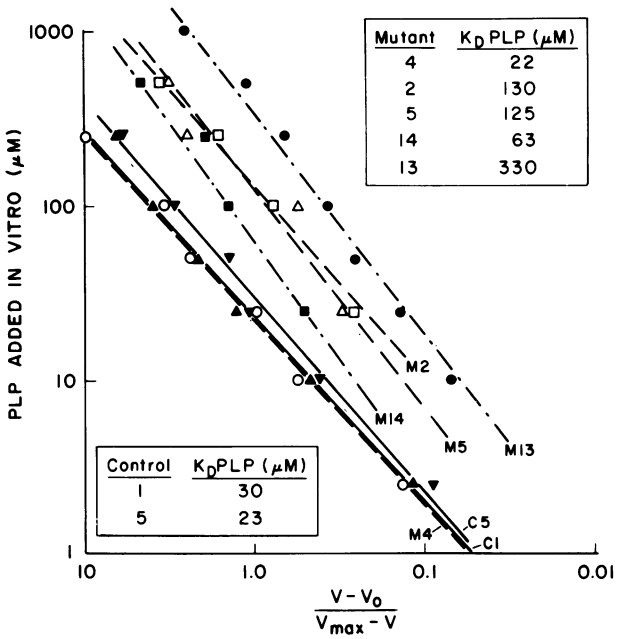

FIGURE 6 Estimation from Hill plots of PLP dissociation constants (K_d PLP) for control (——) and mutant (in vivo pyridoxine-responsive – – –; in vivo pyridoxine nonresponsive — · — ·) CS. Extracts of cultured skin fibroblasts of control 1 (▼) and 5 (▲) and mutants 2 (□) 4 (○) and 13 (●) were prepared as described in the legend to Fig. 5. Extracts of mutants 5 (△) and 14 (■) were prepared by dialysis in 1 mM hydroxylamine for 6 h followed by dialysis in the buffer without hydroxylamine for 18 h. V = velocity at designated PLP concentration; V_{max} = velocity at highest PLP concentration employed; V_o = velocity with no PLP added. K_d PLP was obtained from that concentration of PLP at which $V = V_{max}/2$, i.e., where $(V\text{-}V_o)/(V_{max}\text{-}V) = 1$.

creases of 140–600% on the addition of PLP, compared with more modest increases of <50% in control lines and in four other lines from in vivo pyridoxine-responsive patients. Thus, there was no general correlation in the mutants between the striking in vitro stimulation of CS by PLP and the in vivo response to pyridoxine. We conclude that, as shown originally by Uhlendorf et al. (15), patients who are pyridoxine responsive in vivo tend to have greater residual synthase activity in their cell lines than nonresponsive patients. However, neither basal activity, nor the degree of stimulation of CS by PLP in vitro can distinguish any single in vivo responsive patient from any nonresponsive one.

We have also further examined some of the properties of mutant CS obtained from fibroblasts. Previous studies of such mutant synthases have suggested a reduced affinity for PLP in some fibroblast lines (12, 16). In all such previous studies, however, enzyme preparations contained an appreciable fraction of holo-CS. We prepared apo-CS from control and mutant lines to assess this property more rigorously. As reviewed by Snell (19), widely different conditions have been employed to resolve PLP-requiring enzymes. Extensive attempts at resolution and reconstitution of control CS holoenzyme yielded conditions which gave maximal yields of apoenzyme (>90% of total) and maximal recovery of total synthase activity (65–85% of activity of unresolved synthase) after reconstitution with PLP. Under these conditions, however, CS preparations from mutant lines (apart from mutant 4) showed irreversible loss of activity. The modified resolution conditions used for these mutant lines resulted in a wider range of apo-CS yield (70–98%) but no distinct difference in total enzyme recovery (70–78%).

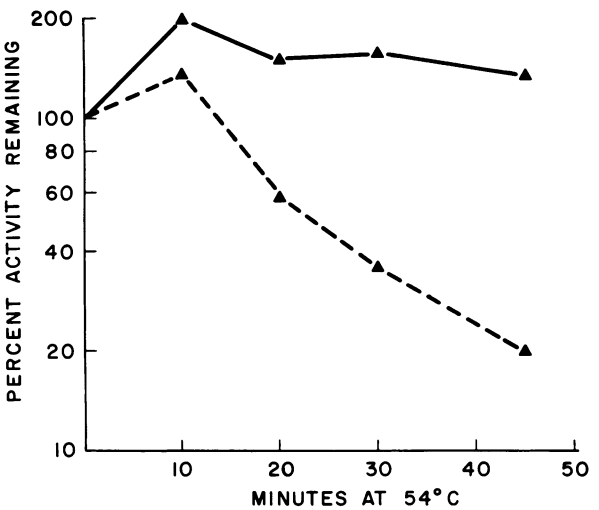

FIGURE 7 Heat stability of apo-CS and holo-CS in extracts of control fibroblasts. Enzyme extract was dialyzed in 1 mM hydroxylamine in 0.05 M potassium phosphate buffer, pH 7.5, for 24 h, followed by dialysis in the same buffer without hydroxylamine for 24 h. Values joined by a broken line were obtained by heating aliquots of apoenzyme extract at 54°C for varying lengths of time, cooling in an ice bath, then assaying with 1 mM PLP. Values joined with a continuous line were from holoenzyme obtained by adding PLP (2 mM) to apo-CS before heating and then assaying with a final PLP concentration of 1 mM. Remaining synthase activity is expressed as a percentage of activity in samples which were not heated. The data presented here are representative of three separate experiments.

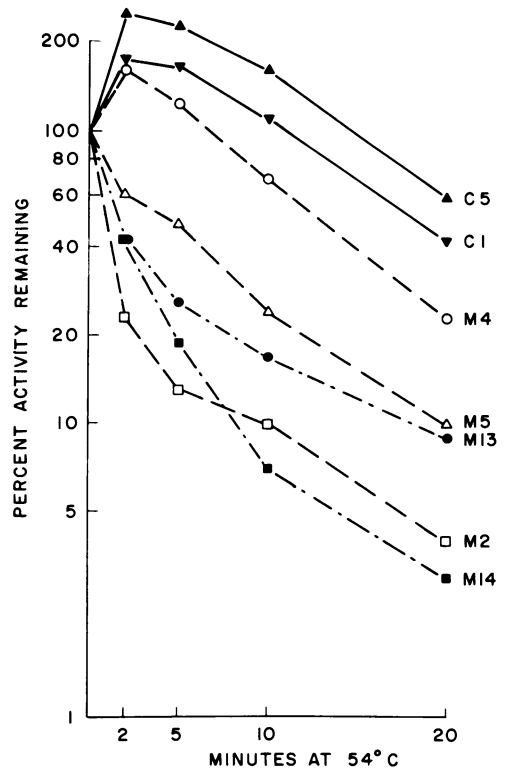

FIGURE 8 Heat stability of CS activity in extracts of control and mutant cultured skin fibroblasts. Extracts were dialyzed for 2 h in 0.05 M potassium phosphate buffer, pH 7.5. Aliquots of the extracts were heated without added PLP for varying lengths of time at 54°C, cooled in an ice bath, then assayed in the presence of PLP (1.0 mM). Remaining synthase activity is expressed as a percentage of the activity in unheated samples. Controls (C1 and C5) and mutants (M2, M4, M5, M13, M14) are designated by the same symbols employed in Fig. 6.

We[2] and others (18) have shown that incubation of partially purified, hepatic CS apoenzyme with high and low concentrations of PLP for 1 h at 37°C achieves equilibrium between apoenzyme and cofactor. We, therefore, employed these conditions when estimating K_d PLP in extracts of control and mutant fibroblasts. It is likely that the absolute estimates of K_d PLP for CS obtained with crude fibroblast extracts (20–30 μM) are

[2] Kraus, J., S. Packman, B. Fowler, and L. E. Rosenberg. Unpublished observations.

significantly higher than values obtained with pure enzyme preparations. Thus, we have observed a K_d PLP of 3 μM for CS purified > 30-fold from human liver.[2] Without regard to the absolute value, the estimates of K_d PLP obtained with crude extracts are reliable for comparative purposes and indicate that one of the mutant lines studied contains an altered CS with reduced catalytic activity but normal affinity for PLP, whereas mutant synthases from other lines have a distinctly reduced affinity for the cofactor.

Thermostability studies of control apo- and holo-CS and of mutant CS provided two interesting findings. First, control apoenzyme exhibited less thermal activation and greater thermolability than did holo-CS. This stabilization of CS by PLP confirms previous observations with crude extracts of human liver (20) and partially purified enzyme from cultured fibroblasts (16), all of which demonstrate that CS behaves in a manner similar to other PLP enzymes (21–25) which can be stabilized in vivo and in vitro by pyridoxine administration or PLP addition. Second, using extracts of mutant and control fibroblasts from which bound PLP was not resolved, the control CS was significantly more thermostable than the mutant enzymes tested. The mutant with a normal K_d PLP exhibited a degree of initial activation and subsequent decay on an intermediate level found between that of control preparations and the other mutants tested. Thus, it appears that those mutants with a much reduced affinity for PLP, and which therefore exist mainly in the apoenzyme form, tend to be the least stable in regard to heating. It is not possible to be certain that this in vitro thermolability reflects a greater degree of intracellular lability in vivo.

It seems likely that in vivo pyridoxine responsiveness in patients whose CS has a reduced affinity for PLP and(or) reduced stability can be accounted for by a partial, perhaps only modest, enhancement of catalytic activity brought about by the vitamin supplement. Clearly, this construct does not explain responsiveness in patients (no. 4) whose CS exhibits essentially normal kinetics for PLP and near normal stability, or nonresponsiveness in patients (nos. 13 and 14) whose CS properties are still indistinguishable from those found in the cells of responsive patients. Other intrinsic properties of CS must be examined to clarify this matter. Tate and Meister (26, 27) showed that catalytic activity of another PLP-dependent enzyme, aspartate β-decarboxylase, was modulated by PLP-induced alteration in subunit interaction. Such modulation could play an even more important role in interaction between mutant subunits. It is, of course, possible that the results of experiments employing cultured cells may not always accurately reflect events in tissues that are more critical in defining the in vivo situation. For instance, variation in any number of steps regulating pyridoxine metabolism—intestinal absorption, binding to plasma proteins, transport into cells, enzymatic conversion to PLP—could account for the failure of patients 13 and 14 to respond to pyridoxine even if their cells contain a mutant enzyme similar or identical to that in cells of other responsive patients. Furthermore, we cannot dismiss the possibility that pyridoxine responsiveness depends on events other than those directly related to modification of the mutant enzyme. Thus, nonenzymatic complexing of PLP and homocysteine or stimulation of alternate pathways of sulfur amino acid metabolism by PLP could explain some of the changes in plasma or urinary amino acid concentrations observed in responsive patients. No evidence for either mechanism currently exists.

Finally, we conclude that the findings presented in this study indicate the existence of at least three general classes of CS mutants: first, those with no detectable synthase activity; second, those with much reduced activity, normal affinity for PLP and normal heat stability; and third, those with marked reductions in activity, affinity for PLP, and heat stability.

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