

Affinity of Cystathionine β -Synthase for Pyridoxal 5'-Phosphate in Cultured Cells: A *MECHANISM FOR PYRIDOXINE-RESPONSIVE HOMOCYSTINURIA*

Mark H. Lipson, ... , Jan Kraus, Leon E. Rosenberg

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Affinity of Cystathionine β -Synthase for Pyridoxal 5'-Phosphate in Cultured Cells

A MECHANISM FOR PYRIDOXINE-RESPONSIVE HOMOCYSTINURIA

MARK H. LIPSON, JAN KRAUS, and LEON E. ROSENBERG, *Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510*

ABSTRACT Previous attempts to correlate in vivo pyridoxine-responsiveness with in vitro assays of cystathionine β -synthase activity in synthase-deficient homocystinuric patients have been only partially successful. All such studies, however, have been conducted with extracts of cultured skin fibroblasts grown in medium containing a high concentration (1,000 ng/ml) of pyridoxal. Having recently shown that such growth conditions may obscure important aspects of enzyme-coenzyme interactions by saturating most synthase molecules with their cofactor, pyridoxal 5'-phosphate, we have established conditions for growth of cells in pyridoxal-free medium. Under these conditions, intracellular pyridoxal 5'-phosphate fell by >95%, and saturation of cystathionine β -synthase apoenzyme with pyridoxal 5'-phosphate decreased from a predepletion value of 70% to <10%. When such depleted cells were grown in media containing pyridoxal concentrations ranging from 0 to 1,000 ng/ml, cellular pyridoxal 5'-phosphate reached a maximum of 30 ng/mg cell protein at a medium pyridoxal concentration of 100 ng/ml. Maximal saturation of apoenzyme with coenzyme in control cells was reached at a medium pyridoxal concentration of 10 ng/ml. In contrast, maximal saturation of residual apoenzyme in cells from an in vivo responsive patient was achieved at a medium pyridoxal concentration of 25–50 ng/ml, whereas that from cells from an in vivo unresponsive patient was reached at 100 ng/ml. Estimates of the affinity of control and mutant cystathionine β -synthase for pyridoxal 5'-phosphate in cell extracts supported the differences observed in intact cells. The apparent K_m of cystathionine β -synthase for pyridoxal 5'-phosphate in extracts of depleted cells from four in vivo-responsive patients was two to four times that of control. In contrast, the K_m for pyridoxal 5'-phosphate in two lines from in vivo

nonresponsive patients was 16- and 63-fold normal. These results suggest that cystathionine β -synthase activity in cells from patients containing a mutant enzyme with a moderately reduced affinity for pyridoxal 5'-phosphate can be increased by pyridoxine supplements in vivo, whereas that from patients whose enzyme has a more dramatically reduced affinity for the coenzyme cannot be so modulated because of limits on the capacity of such cells to accumulate and retain pyridoxal 5'-phosphate.

INTRODUCTION

Homocystinuria caused by deficiency of cystathionine β -synthase (CS)¹ is a well-recognized, autosomal recessively inherited inborn error of sulfur amino acid metabolism. The major clinical manifestations of this disorder include dislocated optic lenses, thrombotic vascular disease, and mental retardation. The most prominent biochemical abnormalities in affected patients are elevated plasma and urinary concentrations of methionine and homocystine, and a much reduced plasma cystine (1). CS (L-serine hydro-lyase [adding homocysteine], EC 4.2.1.22) requires pyridoxal 5'-phosphate (PLP) as a coenzyme. Consequently, many affected patients have been treated with large doses of oral pyridoxine, the vitamin precursor of PLP. In about half of such individuals, sulfur amino acid concentrations in blood and urine have returned to near normal levels. Such patients are classified as in vivo pyridoxine-responsive (2). All attempts to correlate such in vivo responsiveness with stimulation of residual CS activity by PLP in vitro have been less than successful. Uhlendorf et al. (3) found that in vivo responsiveness or nonresponsiveness correlated generally with

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¹Abbreviations used in this paper: C, control; CS, cystathionine β -synthase; MEM, minimal essential medium; N, in vivo nonresponsive; PL, pyridoxal; PLP, pyridoxal 5'-phosphate; R, in vivo responsive.

the presence or absence, respectively, of some residual CS activity in extracts of cultured fibroblasts. Several *in vivo* nonresponsive patients have been described, however, whose cells retained some residual CS activity (3, 4). Kim and Rosenberg (5) reported that CS in fibroblast lines from several *in vivo* responsive patients was more thermolabile than normal, and had a reduced affinity for PLP. Increased thermostability and enhanced catalytic activity were observed in the presence of saturating amounts of the coenzyme. On the basis of these findings, they proposed that *in vivo* pyridoxine responsiveness depended on the quality as well as the quantity of residual enzyme. Exceptions to this thesis were reported in that stimulation of residual CS activity by PLP *in vitro* was sometimes as great in cells from *in vivo* unresponsive patients as from responsive ones (3, 4), and in that apparent K_m of CS for PLP were not reproducibly elevated in cells from responsive patients (4).

All such studies, however, have been performed with extracts of cells grown in medium containing a very high concentration of pyridoxal (PL) (1,000 ng/ml); that is, under conditions in which most (>70%) of the synthase in normal cells exists as holoenzyme rather than apoenzyme. We speculated that such growth conditions might have obscured the very apoenzyme-coenzyme interactions that related *in vivo* responsiveness to *in vitro* enzyme activity. Therefore, we decided to re-examine the matter in cells propagated in PL-free medium. This was accomplished by omitting PL from standard Eagle's growth medium, and by using hydroxylamine to remove virtually all protein-bound PL and PLP from the human serum needed to support fibroblast growth (6). We were able to grow control fibroblasts to confluence in such PL-depleted medium for four serial passages without significant alterations in cell viability or growth rate. Under these conditions, cellular PLP content fell by over 95%, and >90% of all synthase activity was present as apoenzyme.

We have now examined the effect of such depletion on synthase activity in lines from *in vivo* responsive and unresponsive patients with CS deficiency. Our results suggest that *in vivo* responsiveness in individuals with some residual CS activity is related both to the affinity of the mutant apoenzyme for PLP and to the capacity of cells to accumulate PLP.

METHODS

Eagle's minimal essential medium (MEM) prepared with and without PL·HCl was purchased from Gibco Laboratories, Grand Island Biological Co. (Grand Island, N. Y.). Human serum was collected from healthy volunteers. PLP and PL were purchased from Sigma Chemical Co. (St. Louis, Mo.); hydroxylamine hydrochloride from Aldrich Chemical Co., Inc. (Milwaukee, Wis.); L-[U-¹⁴C]serine from New England Nuclear (Boston, Mass.); L-cystathionine and L-homocysteine thiolactone from Calbiochem-Behring Corp., American Hoechst

Corp. (San Diego, Calif.) and L-serine from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.).

Cell culture. Three skin fibroblast lines from controls (lines 86, 87, and 237), four lines from *in vivo* responsive patients with CS-deficient homocystinuria (lines 42, 338, 343, and 676), and two lines from *in vivo* nonresponsive patients (lines 375 and 382) were used in these experiments. Experiments were conducted with cells maintained in culture between 6 and 15 passages. Cells were cultured in 32-oz glass bottles (Bellco Glass, Inc., Vineland, N. J.) at 37°C in a 5% CO₂/95% air atmosphere using Eagle's MEM containing PL at concentrations between 0 and 1,000 ng/ml and kanamycin (100 µg/ml), supplemented with 1% (vol/vol) nonessential amino acids and 10% (vol/vol) human serum.

Resolution of B₆ vitamins from human serum. To attain a nearly vitamin B₆-free system, 50 ml of human serum was dialyzed for 24 h at 4°C against one change of a solution of 2 liters of 5 mM hydroxylamine in phosphate-buffered saline, pH 7.4, followed by dialysis for 24 h against two changes (2 liter each) of the same buffer without hydroxylamine.

Conditions for depletion and repletion. Skin fibroblasts were grown to confluence in Eagle's MEM containing 1,000 ng/ml PL and hydroxylamine-treated human serum. They were then transferred to vessels containing medium with no added PL and hydroxylamine-treated human serum, and grown to confluence for one or two passages. The PLP-depleted cells were then used for two kinds of estimates of affinity of CS for PLP: first, in which CS activity was determined in cell extracts after repletion experiments in medium containing increasing concentrations of PL; second, in which CS activity was measured in cell extracts as a function of PLP concentration added *in vitro*.

Cell extracts. Cells were grown to confluence (3–6 d after subculture), then harvested with 0.1% trypsin solution, washed twice with phosphate-buffered saline (pH 7.4), and then stored as cell pellets at –80°C until assayed. Cell extracts were prepared by sonication in 0.05 M potassium phosphate buffer, pH 7.5, containing 1 mM β-mercaptoethanol. The supernate obtained after centrifugation at 10,000 g for 10 min at 4°C was used for enzymatic and PLP assays.

Enzyme assay. CS activity was assayed as described by Fowler et al. (4). "Holoenzyme" was quantitated by assaying in the absence of added PLP, whereas "total" enzyme was measured in the presence of 1 mM PLP. 1 U of CS is defined as that amount of enzyme that catalyzed the formation of 1 nmol of cystathionine in 1 h at 37°C. Specific activity was expressed as units per milligram of protein determined by the method of Lowry et al. (7).

PLP kinetics. Estimation of CS affinity for PLP was carried out as described previously (4), except that extracts containing CS apoenzyme were incubated with PLP for 30 min at 37°C before addition of substrates.

PLP determination. PLP content was measured by the fluorometric method of Grigor et al. (8) with the following modifications. To samples of fibroblast extracts (250–500 µl), water was added to a total volume of 750 µl. Cold 1.6 N HClO₄ (250 µl) was added and, after mixing, the tubes were allowed to stand on ice for 30 min. The samples were centrifuged at 1,500 g for 5 min and 675 µl of supernate was removed for assay. To each aliquot was added 224 µl of a mixture containing 1.4 N KOH and 0.5 M potassium phosphate buffer, pH 7.0. Then 150 µl of 0.006 N KCN in 0.04 M potassium phosphate buffer, pH 7.0, was added; for each blank, 150 µl of the buffer without KCN was used. The tubes were stoppered and placed in a 50°C water bath for 60 min. After 150 µl of 0.91 N tartaric acid was added, the tubes were centrifuged for 5 min at 1,600 g. Fluorescence was determined with a Perkin-Elmer double beam fluorescence spectrophotometer (Perkin-Elmer Corp., Instrument Div.

(Norwalk, Conn.) using 300 μ l of sample. Excitation was at 320 nm and emission at 420 nm. For each assay, PLP standards containing 1–50 ng of PLP were included. In addition, known quantities of PLP were added to extracts to determine recovery.

RESULTS

CS activity in control and CS-deficient fibroblast lines. Results of multiple determinations of CS activity in extracts of cells grown in standard PL supplemented (1,000 ng/ml) medium are summarized in Table I. Residual CS activity in the six CS-deficient lines ranged from ~1 to 14% of control. Although the ratio of CS holoenzyme/CS total enzyme in the mutant lines (0.18–0.63) was distinctly lower than that in control cells (0.70–0.72), there was no difference between cells from in vivo pyridoxine responsive patients and those from in vivo nonresponsive ones.

Effect of PLP depletion on CS activity. Cells were grown in PL-free medium for four passages and assayed after each passage for PLP content and CS activity. The results for a representative control line are shown in Fig. 1. Cellular PLP content fell rapidly. Typically, after one passage in PL-free medium, cells retained only ~6% of the PLP initially present. Subsequent passages resulted in only modest further losses in PLP content. Depletion of cellular PLP was associated with

a sharp fall in the ratio of CS holoenzyme/total enzyme from 0.7 to <0.1, indicating that, in the PLP-depleted cells, CS was present predominantly in the apoenzyme form. In all mutant lines, one passage in PL-free medium lowered cellular PLP content as in controls and resulted in reducing CS holoenzyme activity to undetectable values (data not shown).

PLP kinetics. Determinations of K_m of apoenzyme for PLP in extracts of PLP-depleted cells from three controls and the six mutant lines are summarized in Table II. K_m values were estimated from Hill plots similar to those shown in Fig. 2 (which depict data from the two control lines having the highest and lowest K_m for PLP, from two of the lines from in vivo responsive patients with the highest and lowest K_m , and from each of the two lines from in vivo unresponsive patients). The apparent K_m of apo-CS for PLP in the three control lines ranged from 52 to 85 μ M (mean 63 μ M). The four lines from in vivo responsive patients had approximately two to four times higher values of 145–200 μ M, whereas the two lines from in vivo nonresponsive patients showed much more elevated K_m values of 990 μ M and 4,000 μ M, ~16- and 63-fold that of the controls.

Effect of PLP repletion on CS activity. To determine whether these apparent K_m differences reflected the situation in intact cells, PLP-depleted cells were

TABLE I
Cystathionine β -Synthase Activity in Control and Mutant Fibroblast Lines

	Cell line number*	CS activity†		
		Holoenzyme	Total enzyme	Holoenzyme Total enzyme
		U/mg		
Clinical classification				
Controls	C-86	9.3	13	0.72
	C-87	12.1	17	0.71
	C-237	8	11.4	0.70
Pyridoxine-responsive homocystinuria	R-42	0.23	1.27	0.19
	R-338	1.2	1.9	0.63
	R-343	0.12	0.61	0.20
	R-676	0.24	0.73	0.33
Pyridoxine-nonresponsive homocystinuria	N-375	0.21	1.15	0.18
	N-382	0.21	0.48	0.44

* Cell lines 42, 338, 343, 375, and 382 were referred to previously as numbers 2, 4, 5, 13, and 14, respectively (4). The prefix letters C, R, and N shown here and in subsequent tables and figures denote lines from control, in vivo responsive, and in vivo nonresponsive patients, respectively.

† CS activity is expressed in units per milligram protein. Holoenzyme was measured in the absence of added PLP, whereas total enzyme was quantitated in assays in the presence of 1 mM PLP. Data shown are the mean of at least three separate determinations on each line.

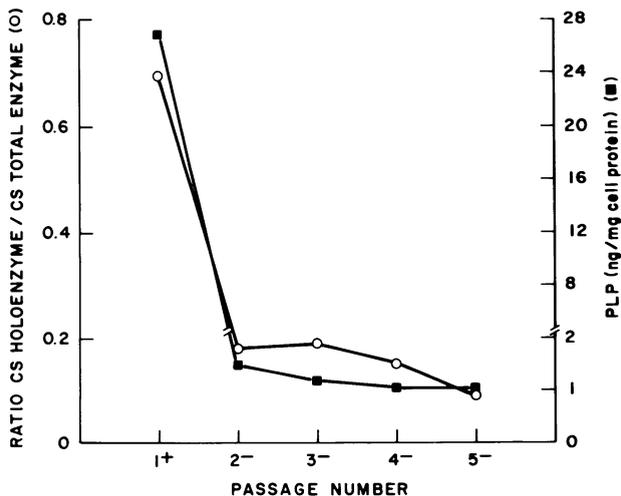


FIGURE 1 Effect of cell growth in PL-free medium on intracellular PLP content and saturation of CS with PLP. Cells were initially grown to confluence in Eagle's MEM containing 1,000 ng PL/ml and hydroxylamine-treated human serum. They were then subcultured and grown in medium containing no PL and hydroxylamine-treated human serum for four additional passages. CS activity was assayed after each passage in cell extracts with (total enzyme) and without (holoenzyme) *in vitro* addition of 1 mM PLP. Intracellular PLP was determined fluorometrically. +, growth in medium containing PL at 1,000 ng/ml; -, growth in medium containing no PL.

transferred for one passage into flasks containing media with concentrations of PL ranging from 0 to 1,000 ng/ml. Such additions of PL to the growth medium led to a rapid increase in cellular PLP content both

TABLE II
Affinity of Cystathionine β -Synthase for Pyridoxal 5'-Phosphate in Fibroblast Extracts

	Cell line number	K_m PLP*
		μM
Clinical classification		
	Controls	
	C-86	52
	C-87	52
	C-237	85
Pyridoxine-responsive	R-42	155
	R-338	145
	R-343	195
	R-676	200
Pyridoxine-nonresponsive	N-375	990
	N-382	4,000

* K_m PLP is defined as that concentration of PLP added *in vitro* which yielded half-maximal CS activity in extracts of control and mutant lines. Data from which such apparent K_m values were derived is shown in Fig. 2.

in control and CS-deficient cells as shown in Fig. 3A. Maximal cellular PLP content was reached at a medium PL concentration of 100 ng/ml; a further 10-fold increase in medium PL concentration did not lead to a further increase in intracellular PLP.

The associated response of control and mutant CS aposynthase to increases in PL concentration in the growth medium and in cellular PLP content is illustrated in Fig. 3B. A clear difference can be seen between the control and mutant lines, and also between the response of *in vivo* responsive (R)-676 and *in vivo* nonresponsive (N)-375. CS holoenzyme activity in the control line roughly paralleled the rise in cellular PLP, reached its maximum value at a PL concentration in the medium of 10 ng/ml, and plateaued thereafter.

The representative mutant cells, however, behaved quite differently. R-676 had no measurable CS holoenzyme at 1 ng/ml of PL; appreciable holoenzyme was noted only at a PL concentration of 10 ng/ml or more. Holoenzyme activity then increased progressively and reached a maximal value at a medium PL concentration of 25-50 ng/ml. The curve in the cells from the nonresponsive patient (N-375) was shifted even further to the right: no holoenzyme activity was

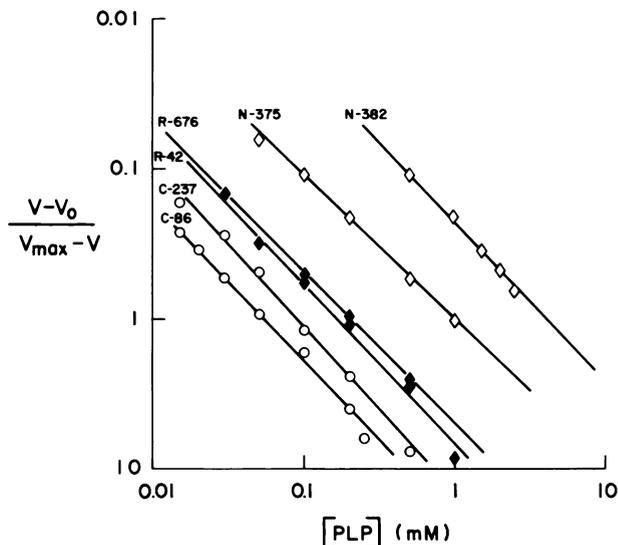


FIGURE 2 Estimation from Hill plots of apparent K_m values of CS for PLP. The designations C, R, and N refer to lines from control, *in vivo* responsive, and *in vivo* nonresponsive patients, respectively. Extracts of PLP-depleted cells were used as source of CS apoenzyme. Cells were depleted of PLP for one passage (mutants) or two passages (controls) as described in the legend to Fig. 1. K_m (PLP) is defined as that concentration of PLP at which $V - V_0 / V_{max} - V = 1$, where V = reaction velocity, V_0 = velocity observed in the absence of added PLP, and V_{max} = maximal velocity extrapolated from velocities observed at increasingly large concentrations of PLP.

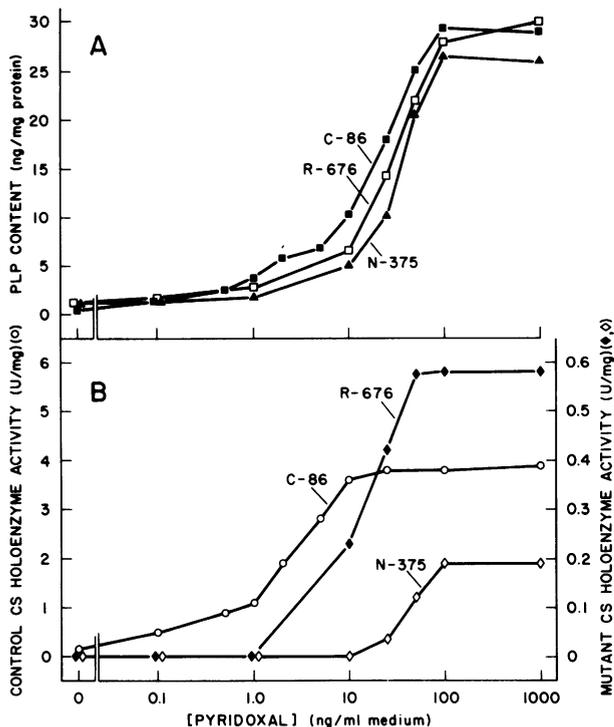


FIGURE 3 Effect of progressive increase in medium PL concentration on intracellular PLP content (A) and on CS holoenzyme activity in PLP-depleted fibroblasts (B). The designations C, R, and N refer to lines from control, in vivo responsive, and in vivo nonresponsive patients, respectively. Cells were depleted of PLP for one passage (mutants) or two passages (control) as described in the legend to Fig. 1. The cells were then subcultured for one passage into flasks containing medium with increasing concentrations of PL ranging from 0–1,000 ng/ml. Holoenzyme was assayed in cell sonicates in the absence of added PLP. The assay for synthase employed was capable of detecting as little as 0.025 U of enzyme activity per milligram of protein. Cellular PLP was estimated in aliquots of cell sonicates using a fluorometric assay. Note the 10-fold difference in scale for control holoenzyme (B, left ordinate) and for mutant holoenzyme (B, right ordinate).

detected below a PL concentration of 10 ng/ml, and maximal activity was seen only at 100 ng PL/ml medium. It is noteworthy that CS holoenzyme did not increase further in either mutant line when the PL concentration was increased above 100 ng/ml, that concentration which produced maximal augmentation of cellular PLP content.

When the holoenzyme activities shown in Fig. 3B are expressed as a percent of total enzyme, that is, as an index of apoenzyme saturation with PLP, the distinct differences between the lines were again clear (Fig. 4). The control line retained significant holoenzyme activity after PLP depletion, whereas the mutants had none; maximal saturation of control apoenzyme with PLP (70% of total) was reached at

10 ng PL/ml. In both mutant lines, the maximal extent of CS saturation with PLP and the medium PL content at which such saturation was achieved were altered. In line R-676, holoenzyme never exceeded 38% of total, and this degree of saturation was achieved at medium PL concentrations of 25–50 ng/ml. In line N-375, holoenzyme reached only 19% of total, and then only at 100 ng PL/ml. No conversion of remaining CS apoenzyme to active holoenzyme was observed in either mutant line beyond that achieved at the medium PL concentration (100 ng/ml) at which the cellular PLP content reached its maximum.

DISCUSSION

The less than satisfactory correlation in CS-deficient homocystinuria between the biochemical response of the patient to orally administered pyridoxine and the response of that patient's CS activity in cell extracts to PLP has puzzled investigators in the field. We have shown recently (6) that the large excess of PL present in the standard growth medium results in a maximal intracellular PLP content and full saturation of certain PLP-dependent enzymes, including CS. Thus, interpretation of studies aimed at defining the affinity of CS for PLP in cells grown under these conditions is exceedingly difficult. Our previous attempts (4) to resolve PLP from mutant CS holoenzyme with hydroxylamine in vitro were largely unsuccessful as a result of the instability of the mutant enzymes. We have, therefore, established conditions for depleting fibroblast cells of PLP "in culture" by growing them in PL-free medium. Cell viability during several passages in this medium was judged normal on the basis of several criteria (6). Our results show that,

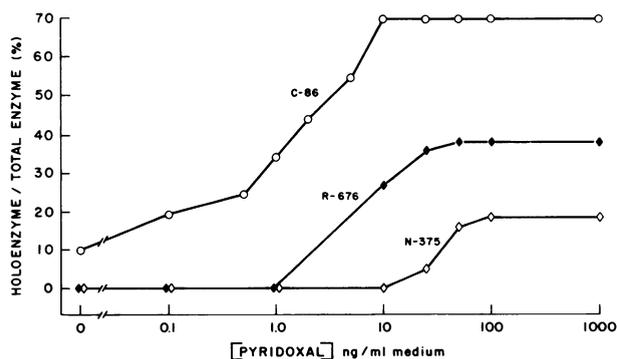


FIGURE 4 Effect of increasing PL concentration in growth medium on saturation of CS apoenzymes with PLP in PLP-depleted fibroblasts. The designations C, R, N, and conditions for depletion and repletion of PLP are the same as in Fig. 3. CS holoenzyme and CS total enzyme were assayed in the absence and presence of 1 mM PLP, respectively. The ratio of holoenzyme/total enzyme determines the extent of apoenzyme saturation with PLP.

in cells depleted in this way, nearly all of the synthase exists as apoenzyme, thereby facilitating in vitro estimates of the affinity of synthase for PLP. Our data suggest, moreover, that CS deficient patients who are responsive to pyridoxine in vivo have an affinity of CS for PLP two to four times less than control. In contrast, those patients with residual CS activity who are not responsive in vivo have a markedly reduced affinity of CS for PLP—from 16–63 times less than normal.

To determine whether these in vitro K_m estimates bore some relationship to events in intact cells, we grew PLP-depleted cells from representative control, responsive, and nonresponsive patients in media containing pyridoxal concentrations from 0 to 1,000 ng/ml, and we estimated PLP content and synthase activity in extracts therefrom. The results showed that holoenzyme formation in the line from the responsive patient occurred at a much higher PL concentration in the medium than that needed for formation of holoenzyme in the control cells, but much lower than that observed for cells of the nonresponsive patient. Furthermore, in the in vivo responsive patient, 38% of synthase activity existed as holoenzyme maximally, whereas in the in vivo nonresponsive subject, only 19% of CS molecules were converted to holoenzyme at maximum levels of PLP in the cells.

When the in vitro kinetic and PLP-repletion data are interpreted together, we believe both the affinity of CS for PLP and the cell's ability to accumulate PLP assume importance in understanding in vivo responsiveness to oral pyridoxine in CS-deficient patients. We suggest that in vivo responsiveness or lack thereof depends on at least three factors: (a) the presence or absence of residual synthase activity in mutant cells; (b) the affinity of mutant synthase for its cofactor, PLP; and (c), as importantly, the cell's ability to accumulate PLP. We propose that those patients whose cells contain a mutant synthase with a moderately reduced affinity for PLP (perhaps two to five times less than control) are able to increase cellular PLP content sufficiently after pyridoxine supplementation so that holosynthase activity rises above that critical value needed to prevent accumulation of homocystine and methionine. Pyridoxine nonresponsiveness, on the other hand, may be observed for one of two general reasons: either because the cell contains no residual synthase activity (a situation which appears to exist in most nonresponsive patients), or because the cell contains a mutant synthase whose affinity for PLP is so reduced (perhaps 20–70 times less than control) that, despite any feasible pyridoxine supplements, the cell is unable to increase PLP content enough to stimulate appreciable formation of synthase holoenzyme. In this study we demonstrate that the

fibroblast cell will not accumulate PLP above a certain limit (~30 ng PLP/mg cell protein at 50–100 ng PL/ml medium) even though the PL concentration in the culture medium is increased to 1,000 ng/ml. It has also been shown that the hepatic content of PLP is controlled principally by the protein binding of this coenzyme and its hydrolysis by a cellular phosphatase. When the amount of PLP exceeds the binding capacity of intracellular proteins and apoenzymes, free PLP is hydrolysed by plasma membrane-associated alkaline phosphatase (9). When cell extracts are prepared and these cellular barriers are obviated, much higher concentrations of PLP can be added, thereby creating the possibility that cells from an in vivo nonresponsive patient may be "responsive" in vitro. This caveat must be kept in mind in attempting to correlate events in intact cells or whole patients with those in cell-free extracts.

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