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Coenzyme protection
cystathionine β-synthase
enzyme activation

homocystinuria
thermostability
vitamin B_6 therapy

Cystathionine β-Synthase Deficiency: A Qualitative Abnormality of the Deficient Enzyme Modified by Vitamin B_6 Therapy

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Summary

The thermostability of cystathionine synthase and the effect of pyridoxal phosphate (PLP) on this thermostability were investigated in extracts of normal human liver and in extracts of liver, both before and during pyridoxine (vitamin B_6) therapy, from members of a family with three clinically and biochemically typical, B_6-responsive, synthase-deficient sibs.

Incubation of crude extracts of normal liver at 55° (preincubation) for 3-4 min before assay consistently resulted in a more than 2-fold increase in specific activity (activation) of cystathionine synthase (Fig. 1). With periods of preincubation longer than 4 min, thermal inactivation occurred. When PLP was added to the preincubation mixture, slightly more activation occurred in the first 3-4 min, and there was no observable loss of activity for an additional 25 min.

The activation phenomenon was not observed in extracts of liver which had been obtained from three synthase-deficient sibs before therapy with vitamin B_6 (Index of activation, Table 1). When extracts of liver obtained during vitamin B_6 therapy were studied, however, significant activation was observed. Synthase activity in extracts of liver from the patients' parents, obligate heterozygotes for synthase deficiency, and from a potentially heterozygous sister demonstrated activation similar to that found in control liver extracts.

With periods of preincubation longer than 5 min, the inactivation of synthase in liver extracts from patients receiving pyridoxine-HCl occurred at the same rate as in liver extracts from heterozygotes and from normal subjects (Index of inactivation, Table 1). PLP completely prevented heat inactivation of enzyme from normal liver.

Speculation

Activation of cystathionine synthase is an expression of the ability of the normal enzyme to undergo heat-induced conformational changes. The failure to activate the abnormal enzyme suggests it has an altered molecular structure.

Cystathionine β-synthase (EC. 4.2.1.22), in the presence of the coenzyme, PLP, catalyzes the condensation of homocysteine and serine to form cystathionine. Synthase deficiency (21), which is inherited in an autosomal recessive manner (7), results in the clinical syndrome of homocystinuria (3, 19). The precise nature of the mutation(s) responsible for the deficiency is unknown. Some patients respond biochemically to massive doses of vitamin B_6 in the form of pyridoxine-HCl (Reference 1; also *cf.* Reference 4) and this response usually is accompanied by a significant increase in hepatic synthase activity (11, 20). The mechanism by which vitamin B_6 effects an increase in synthase activity, however, remains unclear. In general, a genetically determined deficiency of enzymatic activity may result from decreased holoenzyme synthesis (due to decreased apoenzyme or coenzyme formation or to their altered association), from decreased enzyme stability, and/or from decreased efficiency of the enzyme (decreased activity per mg enzyme protein). Consequently, vitamin B_6 may increase the steady state activity of hepatic synthase in B_6-responsive, synthase-deficient patients by affecting one or more of these mechanisms.

Heat stability studies have proved useful in comparing genetically determined variants of an enzyme. In addition, PLP has been shown to retard the *in vitro* inactivation by heat and/or proteases of certain PLP-dependent enzymes (12, 23). In light of this, we have investigated the thermostability of cystathionine synthase, and the effect of PLP on this thermostability, in extracts of normal human liver and in extracts of liver, both before and during vitamin B_6 therapy, from a family with three synthase-deficient sibs. All three sibs are B_6 responsive and are clinically and biochemically typical.

METHODS

Control liver was obtained at autopsy 5-24 hr after death or was part of a biopsy taken during abdominal surgery. Biopsies of liver from members of the affected family were obtained percutaneously with a Menghini needle. Informed consent was obtained in writing. Vitamin B_6 therapy was administered orally for 2-12 weeks before repeat biopsy (*cf.* legend, Table 1).

Liver specimens were homogenized in 7 volumes 0.03 M phosphate buffer (pH 6.9) and centrifuged for 1 hr at 40,000 × *g*. The supernatant solution was used for the enzymatic studies. Partially purified extracts were prepared according to the method of Tallan *et al.* (24). Tissue extracts were incubated at 55° for varying lengths of time before assay (preincubation) in the presence or absence of 1.3 mM PLP (final volume 70 μl). The reaction tubes were then plunged into crushed ice for 15 min before assay at 37°. PLP was added where previously omitted to equalize the concentration present during the assay. Conditions for assaying liver extracts were modified from the method of Gaull *et al.* (10) by incubating at pH 8.4 for 45 min. The assay procedure is based on the direct measurement, on an automatic amino acid analyzer, of cystathionine formed in the reaction.

Protein concentration was determined according to the method of Lowry *et al.* (17). Enzymatic activity is expressed as nanomoles of cystathionine formed per mg protein per hr. When activity is expressed as a percentage, 100% refers to enzyme which was not preincubated (zero time).

RESULTS

Crude extracts of normal liver obtained at autopsy were preincubated at 55° for periods up to 4 min before assay. This consistently resulted in at least a 2-fold increase in specific

activity (activation) of cystathionine synthase (Fig. 1). Preincubation for periods longer than 4 min resulted in a typical curve of thermal inactivation, which, in replicate experiments, appeared to level off at 20–30 min. When PLP was added to the preincubation mixture, somewhat more activation occurred during the first 4 min; no observable loss of activity occurred during the following 25 min. Because of the small amounts of liver obtained with a needle, fixed preincubation times of 3 and 10 min were selected for study of biopsied liver. The 3-min preincubation time was chosen because in preliminary experiments this consistently was the time of maximal activation; the 10-min preincubation time was chosen because it consistently was on the linear portion of the descending curve.

Preincubation of extracts of control biopsy material for 3 min at 55° resulted in a 2- to 3-fold increase in specific activity over the mean specific activity of extracts which were not preincubated (zero time value) (Table 1). After a 10-min preincubation, the specific activity was still higher than at zero time, although substantially decreased from the specific activity observed after the 3-min preincubation. Preincubation for 10 min in the presence of 1.3 mM PLP resulted in activities which were much higher than those observed after a 10-min preincubation without added PLP, indicating protection against heat inactivation. The higher relative specific activity at 10 min in the presence of PLP is also a function of the greater activation achieved in the presence of the coenzyme (see Fig. 1). Liver obtained 5–24 hr after death had lower activity than liver obtained *in vivo*, although the patterns of response to heat were similar.

Extracts of liver from three synthase-deficient sibs obtained before treatment with pyridoxine had specific activities of 16, 14, and 9, respectively (Table 1), 4–7% of the mean control value. The activation phenomenon did not occur in extracts of liver from the two sibs from whom enough liver was obtained for heat stability studies. On the contrary, a lower specific activity was found both after 3-min and after 10-min preincubation than was found without preincubation.

When these studies were performed on extracts of liver obtained from the three affected sibs during vitamin B_6 therapy, the results were strikingly different (Table 1). The specific activity of cystathionine synthase in liver extracts which were not preincubated was found to have increased in *sibs 2* and *3* (from 14 to 24, and from 9 to 19), but not in *sib 1* (who was receiving a smaller dose of vitamin B_6; *cf.* legend, Table 1). In addition, significant activation was now observed in all cases after 3 min of preincubation at 55°. After 10 min of preincubation, synthase activities decreased from those found after a 3-min preincubation.

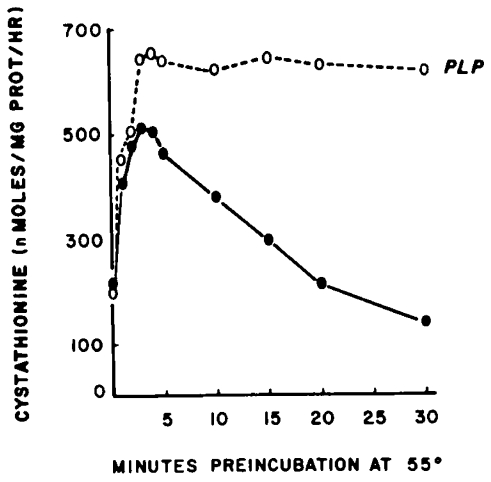

FIG. 1. Effect of preincubation at 55° on specific activity of cystathionine β-synthase in a crude extract of human liver. These data represent one of four replicate experiments, using different enzyme extracts, which showed identical patterns of thermostability. ●——●: preincubation without added pyridoxal phosphate (PLP); O- - -O: preincubation in presence of 1.3 mM PLP.

Table 1. *Heat-induced activation and inactivation of human hepatic cystathionine synthase: Effects of pyridoxine (B_6) in vivo and of pyridoxal phosphate (PLP) in vitro*[1]

Subjects	Cystathionine synthase specific activity[2]	Relative specific activity after preincubation at 55° for time (min) indicated				Index of activation (3/0)[4]	Index of activation (10/3)[5]
		0	3	10	10 + PLP		
Before B_6 therapy							
Controls	225 ± 7[6] (5)	100	263 ± 42[6] (3)	179 ± 14	356 ± 34	2.63	0.70
Affected sibs							
1	16	100	38	60		0.38	[7]
2	14	100	88	89		0.88	[7]
3	9	100					
Obligate heterozygotes							
Mother	88	100	298	154		2.98	0.52
Father	147	100	257	192		2.57	0.75
Potential heterozygote	176	100	264	164		2.64	0.62
After B_6 therapy[8]							
Affected sibs							
1	15	100	210	151		2.1	0.74
2	24	100	174	126		1.74	0.72
3	19	100	137	61		1.37	0.45
Obligate heterozygote							
Father	199	100	233	128		2.33	0.55

[1] Numbers in parentheses refer to number of subjects studied in duplicate. Control liver was obtained from specimens taken from open biopsies which were clinically indicated and in which clinical decision the investigators did not participate.

[2] Nanomoles of cystathionine per mg protein per hr, equal to value without preincubation at 55° (zero time value).

[3] Percentage of zero time value.

[4] Specific activity after a 3-min preincubation at 55° divided by the specific activity without preincubation at 55° (zero time value).

[5] Specific activity after a 10-min preincubation at 55° divided by the specific activity after a 3-min preincubation at 55°.

[6] Mean ± SEM.

[7] A comparison of these data with other values is not valid because of the absence of activation in untreated patients.

[8] Pyridoxine-HCl was administered orally to *sib 1* (50 mg/day) for 12 weeks, to *sibs 2* and *3* (250 mg/day) for 2 weeks, and to the father (250 mg/day) for 8 weeks before biopsy.

Synthase activities in liver extracts from the patients' parents, obligate heterozygotes for synthase deficiency, were intermediate between those found in extracts of normal liver and those found in extracts of liver from the patients (39% of mean control in the case of the mother and 65% in the case of the father). A 3-min preincubation resulted in an activation similar to that seen in extracts from normal liver. After a 10-min preincubation, there was a decrease in specific activities of synthase from those found after a 3-min preincubation. Extracts of liver from the father, obtained during vitamin B_6 administration, had a higher specific activity of synthase than was present in a similar extract obtained before administration of vitamin B_6. The response of synthase activity to preincubation at 55° was again entirely similar to the response of synthase activity in extracts of normal liver. The specific activity of synthase in extracts of liver from the potentially heterozygous sister of the affected sibs was 176 (77% of the mean control value) and the response of the enzyme extract to heat was similar to that of the control subjects.

DISCUSSION

A genetically determined enzymatic deficiency may result from the alteration of any one of a complex series of reactions: apoenzyme synthesis, coenzyme formation and transport, apoenzyme-coenzyme association, and degradative processes. A presumed decrease in coenzyme formation, transport, or association with apoenzyme has been the rationale behind megatherapy with a number of vitamins, including the B_6 vitamins (which most likely act through the coenzymatically active form, PLP). Vitamin B_6, however, may affect enzymatic activity in other ways: *e.g.*, pyridoxine induces rat liver tyrosine α-ketoglutarate transaminase activity *in vivo* by increasing the rate of enzyme synthesis (12).

The mechanism by which massive doses of pyridoxine increase the steady state activity of cystathionine synthase in some patients with B_6-responsive synthase deficiency (11, 20) is still unclear. Our data confirm and extend previous reports (14, 20) of an *in vitro* protective effect of PLP against heat inactivation and demonstrate the magnitude of this effect (Fig. 1). Such protection may reflect a decreased dissociation of coenzyme and apoenzyme in the presence of exogenous PLP, thereby maintaining the holoenzyme in its active and most stable molecular conformation (16). We have found that PLP completely prevents any loss of hepatic synthase activity due to heat denaturation during a 30-min exposure at 55° (Fig. 1). Mudd *et al.* (20) have reported that PLP is as effective in preventing heat inactivation of enzyme from patients as it is in preventing heat inactivation of enzyme from normal subjects and from heterozygotes. Because of the small amount of liver obtained from our patients, we were unable to investigate this point.

A clear distinction between normal and abnormal synthase is found in the ability of normal synthase to undergo heat-induced activation, a property of cystathionine synthase demonstrated for the first time in the present study. The activity of normal enzyme, as well as that of enzyme from obligate heterozygotes, is strikingly increased by preincubation at 55° for 3 min. Such preincubation, on the contrary, decreases the activity of the enzyme from the patients (Index of activation, Table 1). If we assume that activation is an expression of the ability of normal synthase to undergo heat-induced conformational changes, the failure to activate demonstrated by the abnormal enzyme is an indication of an altered molecular structure.

The occurrence of increased enzymatic activity after exposure to heat is unusual, but not unique, among human enzymes. Similar results have been reported for phosphoglucomutase (18) and mannosidase (2). Heat-induced activation of cystathionine synthase appears to be a general characteristic of the enzyme, for we have demonstrated it in the following crude extracts: cultured human skin fibroblasts, cultured lymphoid cell lines, human brain, and liver and brain of rat and monkey (8). It is present also in partially purified extracts of human liver. Heat-induced activation of cystathionine synthase was not reported by other workers (14, 20); however, our experiments are not completely comparable with theirs. Activation of cystathionine synthase by trypsin (20), by *S*-adenosylmethionine (6), and by *S*-adenosylhomocysteine (5) have been reported. The relationship of these phenomena to the heat-induced activation is being investigated in our laboratory.

Although the mechanism of heat activation is unclear at the present time, two explanations seem possible. Enzymatic activity depends to a great extent upon the structure of the enzyme protein, and heat-induced conformational changes in this structure thus may affect activity. In particular, subunit association and dissociation may have profound effects on activity. For example, cold-induced inactivation of pyruvate carboxylase results from dissociation of the enzyme protein into subunits (22, 26); subsequent heat treatment restores enzymatic activity by allowing reassociation of the subunits to tetramers. There is evidence that the cystathionine synthase from rat liver exists as a tetramer (15). Whether this is also true of the human liver enzyme is not known, but the finding that obligate heterozygotes for synthase deficiency generally exhibit much less than the 50% of the mean control value expected on the basis of a simple gene-dose relationship suggests that the human enzyme protein is polymeric rather than monomeric (9, 25). In that case, heat-induced changes in the conformation of the synthase molecule could affect subunit association and the spatial arrangement of binding sites and thereby increase activity by, for example, increasing affinities for coenzyme and/or substrates. An alternative explanation of the activation would involve the release or destruction of an inhibiting substance, resulting in an apparent activation of the enzyme. In this context, a heat-labile enzyme that specifically degrades the apoproteins of PLP-dependent enzymes has been reported to occur in small intestine and in skeletal muscle of the rat (13).

Irrespective of the mechanism of activation, the difference in the response to heat of synthase from affected invididuals appears to be a property by which normal enzyme and that from deficient individuals can be distinguished qualitatively. In addition, *in vivo* administration of vitamin B_6 in the form of pyridoxine-HCl, which in these responsive patients increased hepatic cystathionine synthase activity and ameliorated the biochemical abnormalities in plasma and urine, has been shown to restore the potential for activation to the patients' enzyme. Thus, the *in vivo* therapy with vitamin B_6 does have some, as yet unexplained, effect on the *in vitro* behavior of cystathionine synthase.

CONCLUSION

Heat-induced activation of human cystathionine synthase was demonstrated in extracts of liver from normal control subjects. This property was lacking in enzyme from the synthase-deficient individuals who were studied before administration of pyridoxine. Restoration of the activation phenomenon was achieved by pyridoxine administration *in vivo*, which, in these responsive patients, also alleviated the biochemical abnormalities of the disease.

Heating enzyme preparations for more than 4 min resulted in inactivation. The addition of PLP to normal enzyme before heating completely protected it from inactivation for the duration (30 min) of the experiment.

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Congenital abnormalities
newborn
palmar crease

Palmar Crease Variants and Their Clinical Significance: A Study of Newborns at Risk

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Summary

An analysis of palmar crease variants was carried out in a group of "at risk" newborns, without any evident congenital anomalies. This group consisted of 108 prematures, 74 infants who were small for gestational age, 62 newborns with history of gestational complications, and 46 newborns with a history of intrauterine methadone exposure.

A system of classification was developed based on observations of 500 normal newborns as control subjects, 466 normal mothers, and 200 normal children. The palmar crease variants can be divided into four main groups, schematically presented as normal variants, simian crease and its variants, Sydney line and its variants, and another group of unusual variants which do not fit into the other groups. A study of these groups revealed that familial components, race, sex, and age are factors that can influence the expression of palmar crease patterns. There is an increased frequency of abnormal creases in each of the groups of "at risk" newborns. Moreover, there is an apparent association of interrupted transverse creases and intrauterine methadone exposure.

Speculation

Our findings suggest that examination of palmar creases and the demonstration of variant patterns may provide a useful, objective indicator of possible abnormal fetal development. Since it is important to utilize a standard scheme in routine newborn examination, both to identify palmar crease variants and to establish a baseline for comparative studies, a system for classification of palmar creases is presented.