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Coenzyme protection
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enzyme activation

homocystinuria
thermostability

Cystathionine β-Synthase Deficiency: Differences in Thermostability between Normal and Abnormal Enzyme from Cultured Human Cells

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Summary

The thermostability of cystathionine β-synthase and the effect of pyridoxal phosphate (PLP) and other B_6 vitamers on this thermostability were studied in extracts of cultured skin fibroblasts from normal subjects, from heterozygotes for synthase deficiency, and from patients with homocystinuria due to synthase deficiency.

Incubation of crude extracts of normal fibroblasts at 55° (preincubation) for short periods prior to assay consistently resulted in an increase of cystathionine synthase activity (activation) that reached a maximum at 2 min (Fig. 1). Further preincubation resulted in inactivation. Addition of 0.4 mM PLP to the preincubation mixture resulted in greater activation that reached a maximum at 3-5 min. The subsequent inactivation phase proceeded at a slower rate than had occurred in the absence of PLP, resulting in a doubling of the half-life of thermal inactivation at 55°. PLP was the only one of the B_6 vitamers to have a protective effect.

No significant activation of synthase was observed when extracts of fibroblasts from synthase-deficient patients were preincubated at 55°. Cells from eight patients were studied: extracts from those of four patients demonstrated activity too low to permit measurement of thermostability; extracts of cells from two patients were less thermostable than those from control subjects and showed a protective effect of PLP; extracts of cells from two other patients (sibs) showed very high heat stability in the absence of PLP, but significant inactivation of synthase in the presence of the coenzyme (Fig. 2, *A* and *B*).

Three patterns of response to heating were observed with extracts of fibroblasts from seven obligate heterozygotes (Fig. 3): 1) no activation; 2) activation with and without prior addition of PLP to the preincubation mixture; and 3) activation only in the presence of PLP. The half-life of inactivation at 55° of the extracts from heterozygotes was similar to that seen in extracts from control subjects.

Synthase from cultured long term lymphoid cells also showed activation followed by inactivation when preincubated at 55°, as well as protection by PLP.

Speculation

Heat-induced activation of cystathionine synthase is a physical characteristic of the normal enzyme molecule which is altered in enzyme from patients and from some heterozygotes for synthase deficiency. Although *in vivo* vitamin B_6 therapy restores the ability of hepatic synthase from patients to be activated, this restoration appears not to be the result of a direct stabilizing effect of the coenzyme PLP on the apoenzyme molecule. Furthermore, since PLP does not consistently protect enzyme from patients against heat inactivation *in vitro*, the increase of hepatic synthase activity observed in some patients receiving megavitamin therapy may not be mediated by a protective or stabilizing effect of the coenzyme.

The precise nature of the mutation(s) responsible for cystathionine β-synthase deficiency is not known. However, we recently demonstrated a qualitative difference in a physical property of hepatic synthase from affected individuals, *i.e.*, the response to heat, which is corrected by vitamin B_6 therapy (10). Extracts of liver from normal individuals had increased cystathionine synthase activity after brief incubation at 55° before assay (preincubation). Heat-induced activation was observed also in extracts of liver obtained from heterozygotes for synthase deficiency, but occurred in extracts from patients only during vitamin B_6 therapy. These findings suggested that in patients with homocystinuria due to cystathionine synthase deficiency this enzyme is structurally altered. Whether or not the mechanism of action of the vitamin involved a direct effect on the synthase molecule remained unanswered.

These results led us to investigate further the thermostability of cystathionine synthase and the effects of the *in vitro* addition of PLP, the coenzymatically active form of vitamin B_6, using cultured skin fibroblasts from normal subjects, heterozygotes, and patients with cystathionine synthase deficiency.

MATERIALS AND METHODS

Skin from the inner surface of the forearm was taken for culture. Informed consent was obtained in writing. Fibroblast cultures were established and maintained according to standard tissue culture procedures. Standard growth medium for diploid cells containing 0.5 mg pyridoxine and 0.5 mg pyridoxal/liter was used for most studies. In some experiments medium lacking both of the B_6 vitamers was used; in other experiments the medium was supplemented with 50 or 100 mg pyridoxine/liter. Care was taken to assure that all cultures were harvested at the same stage of confluence. After reaching confluence the cultures were fed, and harvested 3 days later. Lymphoid cell lines were established according to the technique of Beratis and Hirschhorn (1). Cell pellets were washed with saline and stored in liquid nitrogen until assayed, at which time they were lysed in distilled water using a Branson Sonifier with microtip.

Cell extracts were incubated before assay (preincubation) at 55° (or other temperatures as noted) for varying lengths of time either with or without 0.4 mM PLP (final volume 270 μl). PLP was then added where previously omitted in order to equalize the concentration present during the assay. The assay procedure is based on the direct measurement, on an automatic amino acid analyzer, of cystathionine formed in the reaction (4). The assay

conditions for maximal enzymatic activity of cultured cell extracts have been reported previously (3).

Enzymatic activity is expressed as nanomoles of cystathionine formed/mg protein/h. Protein concentrations were determined according to the method of Lowry *et al.* (11).

RESULTS

Incubation of normal fibroblast lysate at 55° for short periods of time prior to assay (preincubation) resulted in an increase of cystathionine synthase activity that reached a maximum at 2 min (Fig. 1). After this initial "activation" phase, activity decreased with further preincubation. In the presence of 0.4 mM PLP, preincubation resulted in activation with a maximum at 3–5 min. The subsequent inactivation proceeded at a slower rate than in the absence of PLP, resulting in a half-life of thermal inactivation of the enzyme in the presence of PLP that was twice that observed without addition of the coenzyme (Fig. 1, inset). The protective effect of PLP also is shown by the residual activity after 15 min of preincubation at 55°, which is twice as high in the presence of PLP as it is in its absence.

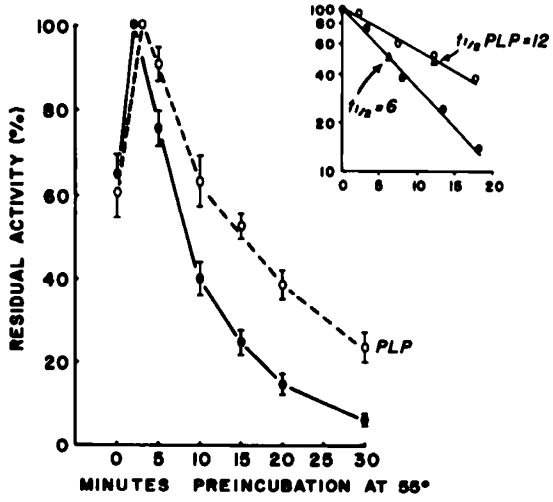

Fig. 1. The effect of preincubation at 55° on the activity of cystathionine synthase in extracts of cultured human skin fibroblasts; 100% represents the maximal activity. ●: preincubation without added PLP; ○: preincubation in presence of 0.4 mM PLP. The inset shows the half-life (in minutes) of thermostability (▲) of the enzyme preincubated at 55° with and without addition of 0.4 mM PLP. The abscissa of the inset represents minutes from the point of maximal activity.

Lysate from normal fibroblasts was preincubated at 55° for 15 min in the presence of each of the B_6 vitamers. The only vitamer to show a significant protective effect was PLP. None of the vitamers, including PLP, had any effect on the specific activity (0 time value) of the enzyme from any of the subjects.

Preincubation of the control fibroblast lysates at temperatures below 55° resulted in less well defined patterns of activation and inactivation. Preincubation at 65° produced immediate inactivation of the enzyme.

When lysates of fibroblasts derived from synthase-deficient patients were preincubated under the same conditions as those used for lysates from controls, no significant activation was observed. Fibroblast extracts from eight patients were studied: four (two responsive to B_6, two unresponsive to B_6) had such low enzymatic activity (less than 0.6 nmol/mg protein/hr) that no conclusions could be drawn regarding thermostability. Of the remaining four (all responsive to B_6), one was less heat stable than the control subjects (Fig. 2*A*), both without and with addition of PLP to the preincubation mixture (half-lives during thermal inactivation of 4.5 and 5.5 min, respectively, as compared to 6 and 12 min, respectively, in control subjects). The protective effect of PLP was shown by a residual activity after 15 min of preincubation which is twice as high in the presence of PLP as it is in its absence. Results with extracts of fibroblasts from a second patient were similar. However, extracts of fibroblasts from the other two patients (sibs) had strikingly different properties in two respects (Fig. 2*B*). First, the enzyme was extremely heat stable, retaining 80% of the initial activity after 10 min of preincubation at 55°. Second, when PLP was added before preincubation, the enzyme was less thermostable, the activity decreasing sharply to less than 10% of the initial value after 10 min at 55°.

With fibroblast extracts from seven unrelated heterozygotes (Fig. 3), three patterns of heat response were observed. In one type no activation was observed; in the second type activation was observed with and without addition of PLP to the preincubation mixture; in the third type activation was observed only in the presence of PLP. The half-lives of thermostability of the enzyme and the activities remaining after 15 min of preincubation, both with and without the addition of PLP, were similar to those found in the control subjects.

When cells were grown in medium deficient in pyridoxine or supplemented 100- or 200-fold the concentration normally present, cell growth was not affected, and neither a change in enzymatic activity nor a different thermostability pattern was observed with cell extracts from any of the subjects.

Synthase activation followed by inactivation after preincubation at 55° was observed also in lysates of normal long term

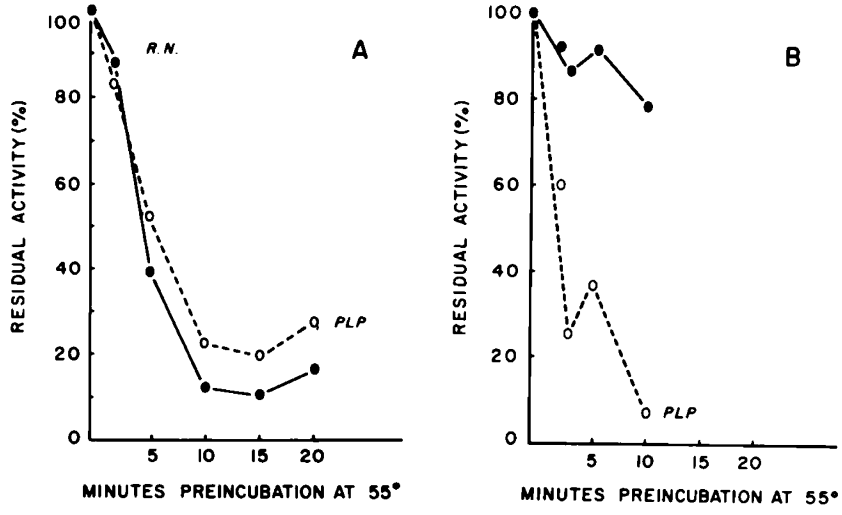

Fig. 2. Thermostability of cystathionine synthase in extracts of skin fibroblasts from two patients with synthase deficiency. ●: preincubation at 55° without added PLP; ○: preincubation in the presence of 0.4 mM PLP.

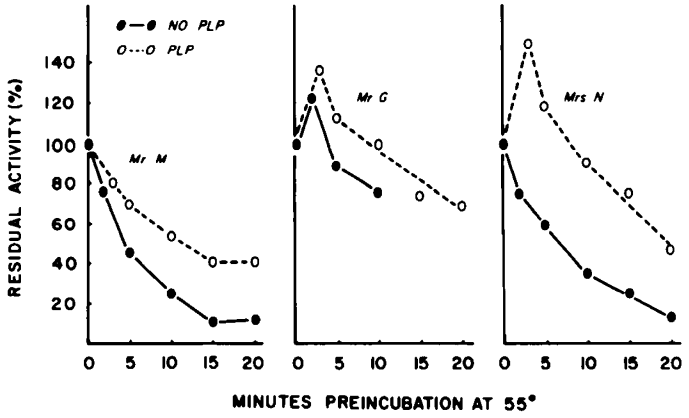

Fig. 3. Thermostability of cystathionine synthase in extracts of skin fibroblasts from heterozygotes for synthase deficiency. ●: preincubation at 55° without added PLP; O: preincubation in the presence of 0.4 mM PLP.

lymphoid cells. Addition of 0.4 mM PLP resulted in greater activation and an increased thermostability of the enzyme.

DISCUSSION

We recently have reported the heat-induced activation of cystathionine synthase in extracts from normal human liver (10). This phenomenon occurred also in extracts of liver from heterozygotes for synthase deficiency, but could be observed in extracts from pyridoxine-responsive patients with synthase deficiency only during megavitamin treatment. We suggested that activation represents an expression of the ability of the normal enzyme to undergo heat-induced conformational change (10). The inability of the abnormal enzyme to be activated by heat suggested an altered molecular structure, and was thought to be a property by which normal enzyme and that from deficient individuals can be distinguished qualitatively. Moreover, *in vivo* administration of vitamin B_6, in restoring the activation phenomenon, appeared to have an effect on the *in vitro* behavior of the enzyme.

We continued our investigations in cultured skin fibroblasts because of their greater availability. Enzyme from normal fibroblasts was activated by heat, whereas enzyme from patients' fibroblasts was not. Since it had been shown that activation by heat could be observed in hepatic extracts from patients only during pyridoxine therapy, the effect of PLP (the coenzymatically active form of pyridoxine) on enzyme from patients was investigated. Even with prior addition of PLP to the preincubation mixture, however, activation of synthase was not observed in fibroblast lysates from any of the patients. Thus, the restoration of heat-induced activation produced in hepatic synthase of patients by pyridoxine therapy *in vivo* is not mediated by a direct effect of PLP on the enzyme molecule. Either synthesis of new synthase molecules, which have the capability to be activated by heat, occurred during B_6 treatment, or such treatment affected another reaction which, in turn, mediated the activation. Either of these general possibilities could account for the difference in this regard between the effects of *in vivo* B_6 therapy and *in vitro* addition of PLP.

After the initial activation phase, synthase from fibroblasts both of normal subjects and of heterozygotes undergoes inactivation upon continued preincubation at 55°. This process of heat-induced inactivation can be retarded greatly by addition of PLP before preincubation. There was sufficient synthase activity in extracts of fibroblasts from four patients to permit investigation of thermostability; two different responses to heating were observed. Enzyme extracts from two unrelated, B_6-responsive patients were less thermostable than enzyme extracts from control subjects, as demonstrated by the shorter half-life of thermostability of patients' enzyme. The increased thermolability found in these two patients resembles that found by Kim and Rosenberg (8) in their case. The residual activity after 15 min of preincubation at 55° was doubled by addition of PLP to extracts of fibroblasts from these two patients. Two other patients, B_6-responsive sibs, demonstrated a different and quite unexpected, response to heating. Enzyme extracts from both sibs possessed great heat stability, demonstrating only 20% loss of activity during 10 min of preincubation at 55°. Even more surprising was the finding that addition of PLP before preincubation actually accelerated the heat-induced inactivation, rather than protecting against it. The variable responses to heat of synthase and the effect of PLP on this response provide further evidence of the genetic heterogeneity of synthase deficiency. Such heterogeneity might explain the apparent discrepancy between the present results (that addition of PLP has the same or greater effect on synthase from normal subjects than on synthase from patients) and those of Kim and Rosenberg (8) (that PLP has a greater protective effect on synthase from patients than on synthase from normal subjects).

The observation of different patterns of response to heat within the heterozygote group is further evidence of genetic heterogeneity in synthase deficiency (5). There is not yet enough data from complete families to judge whether or not any correlation can be made between heterozygotes and homozygotes from the same family. This variation in response to heat is consistent with other lines of evidence that hepatic cystathionine synthase, both in the rat and in man, is not a monomer (6, 9). Furthermore, it is known that heterozygotes for synthase deficiency demonstrate only 20–25% of the mean control activity (2, 3, 5, 15), rather than the 50% expected on the basis of the simple gene-dose relationship observed with monomeric enzymes. The present results lend further support to the suggestion (3, 15) that in the heterozygote the normal polypeptide chains are influenced by the abnormal polypeptide chains via combination into multimeric enzyme units. With such multimeric enzymes, whether they represent the products of multiple alleles or multiple loci for synthase, a broad spectrum of response to heat might be anticipated.

Since megavitamin therapy with pyridoxine restores the activation phenomenon in liver extracts from B_6-responsive patients (10), and PLP does not have this effect *in vitro* on fibroblasts, we investigated the effects of the other B_6 vitamers on synthase thermostability. Activation was not observed in extracts of patients' fibroblasts, either unsupplemented or with addition of any of the B_6 vitamers. As expected, activation was shown by normal fibroblast extracts in the absence of B_6, as well as in the presence of any of the six vitamers. The only vitamer to offer protection against heat inactivation was PLP.

Heat-induced activation of cystathionine synthase has not been reported by other workers who have examined the effects of heat on cystathionine synthase (7, 8, 12); however, their experiments and ours were done in different ways. The studies of Mudd *et al.* (12) were carried out at a temperature of 59° with liver extracts pretreated with trypsin in order to activate the synthase; either or both factors may account for the absence of heat activation in their experiments. Kim and Rosenberg (8) conducted thermostability studies on fibroblasts, using partially purified extracts. Although we did not attempt to purify extracts of cultured skin fibroblasts, activation was present in partially purified (14) extracts of human liver (10). Recently, Griffiths and Tudball (7) have reported the effect of temperature on cystathionine synthase activity in extracts of cultured fibroblasts. They preincubated for a minimum of 2 hr, rather than the 3–5 min necessary for maximal heat activation; therefore, enzyme activation could not have been seen.

Griffiths and Tudball (7) report that 100 mg pyridoxine/liter of culture medium prevented cell growth, but that 25–50 mg/liter resulted in increased synthase activity in cells from their patient (25 mg/liter resulted in slightly increased activity in cells

from their control subject). These results are not consistent with those we have presented here and are not consistent with those of other investigators (13). Griffiths and Tudball (7) present evidence from only one normal and one abnormal cell line; therefore, the discrepancy could be an apparent difference due to genetic heterogeneity.

CONCLUSION

Heat-induced activation appears to be a general characteristic of cystathionine synthase from various tissue sources, as we have demonstrated activation of cystathionine synthase in partially-purified extracts of human liver, in crude extracts of human liver, brain, cultured skin fibroblasts, and lymphoid-cell lines, and in crude extracts of liver and brain of rat and monkey (10, unpublished observations). This phenomenon does not occur in extracts of liver or of cultured skin fibroblasts from untreated patients with synthase deficiency, suggesting a structural alteration in the enzyme synthesized by the mutant gene. Since vitamin B_6 therapy restores activation in hepatic synthase of patients, whereas the B_6 vitamers do not have this effect upon addition *in vitro*, it would appear that this phenomenon is not the result of a direct effect of the coenzyme on the apoenzyme molecule. Pyridoxal phosphate, but no other B_6 vitamer, increases the thermostability of cystathionine synthase from fibroblast lysates of normal individuals and from some, but not all, synthase-deficient individuals. This is further evidence of genetic heterogeneity in this disease.

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