

δ -Aminolevulinic Acid Synthetase in Erythroblasts of Patients with Pyridoxine-responsive Anemia

HYPERCATABOLISM CAUSED BY THE INCREASED SUSCEPTIBILITY TO THE CONTROLLING PROTEASE

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ABSTRACT Properties of δ -aminolevulinic acid synthetase in erythroblasts of patients with pyridoxine-responsive anemia were investigated with special reference to the protease in mitochondria of erythroblasts. δ -Aminolevulinic acid synthetase activity in erythroblasts of patients with this disease before treatment was extremely decreased, whereas it gradually increased in parallel with the improvement of anemia by the therapy with pyridoxal phosphate. The amount of apo- δ -aminolevulinic acid synthetase in erythroblasts before treatment was also extremely diminished. Apparent affinity to pyridoxal phosphate of the apo- δ -aminolevulinic acid synthetase obtained from erythroblasts of the patients was almost the same as that of normal controls. The activity of a new protease which is considered to be engaged in the regulation of δ -aminolevulinic acid synthetase levels in mitochondria of erythroblasts was shown to be in normal range in erythroblasts of the patients. On the other hand, apo- δ -aminolevulinic acid synthetase obtained from the patients was extremely sensitive to the protease. These results indicate that disturbance of heme synthesis characteristic to pyridoxine-responsive anemia could be ascribed to the hypercatabolism of δ -aminolevulinic acid synthetase caused by the increased susceptibility to the controlling protease in erythroblasts.

INTRODUCTION

It is well-known that deprivation of vitamin B₆ from animals causes disorders of erythropoiesis character-

ized by hypochromic, microcytic anemia, elevation of serum iron with increased saturation of iron binding capacity, erythroid hyperplasia in the bone marrow, and increased iron storage in body tissues. These observations indicate that vitamin B₆ is essential to the normal erythropoiesis.

In 1956, Harris et al. (1) reported that a 27-yr-old male who developed hematological symptoms closely resemble those of pyridoxine-deficient animals responded markedly to the treatment with a pharmacological dose of pyridoxine. After this case report a considerable number of patients with pyridoxine-responsive anemia was reported. Besides the patients with classical type, cases with somewhat different features were also reported (2, 3).

Although one case was reported of an 8-yr-old male who developed anemia by dietary deprivation for 130 d and promptly improved by the administration of pyridoxine (4), deprivation of vitamin B₆ and addition of the antagonist, 4-desoxypyridoxine, to the diet of human adults seldom caused hematological abnormalities, in spite of the appearance of other symptoms of pyridoxine deficiency (5). Also, prolonged therapy of patients with lung tuberculosis with isonicotinic acid hydrazide, the antagonist to pyridoxal phosphate, seldom induced anemia, although other symptoms of vitamin B₆ deficiency frequently developed (6). It seems curious that in spite of remarkable response to pyridoxine, symptoms of body wide deficiency of vitamin B₆ (i.e., glossitis, dermatitis, or neuropathy) never develop to the patients with pyridoxine responsive anemia of classical type (3). These facts, with the phenomenon that extremely large doses of vitamin B₆ are required to maintain nearly normal hemoglobin

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levels of the patients, indicate that pyridoxal phosphate in erythropoietic tissues, especially of human beings, plays somewhat different roles than that in other tissues.

In erythropoietic tissues pyridoxal phosphate is known to play a fundamental role in heme synthesis. It is the cofactor of δ -aminolevulinic acid (ALA)¹ synthetase that catalyzes the condensation between succinyl-CoA and glycine to form ALA (7). ALA synthetase is known as the rate-limiting enzyme in heme synthetic pathway (8). Almost all hematological features characteristic to pyridoxine-responsive anemia could be ascribed to the disturbance of heme synthesis in erythroblasts (9). ALA synthetase activity in erythroblasts of patients with primary sideroblastic anemia including those with pyridoxine-responsive anemia was already shown to be inevitably decreased (9). Therefore, decrease of this enzyme activity in erythroblasts of patients with pyridoxine-responsive anemia seems to possess a special importance to its pathogenesis.

Recently, we found a new protease in mitochondria of bone marrow cells including both erythroblasts and granulocytes (10). It rapidly inactivated the apo-form of certain pyridoxal enzymes such as apo-ornithine aminotransferase, apo-homoserine deaminase, apo-serine dehydratase, and apo-ALA synthetase. The protease in erythroblasts that is located on the inside of inner mitochondrial membrane is considered to be engaged in the regulation of ALA synthetase levels by degrading apoform of the enzyme (10). Taking into consideration the facts that one of the most characteristic biochemical features to pyridoxine-responsive anemia is the disturbance of heme synthesis caused by the decreased activity of ALA synthetase in erythroblasts, and that a large amount of pyridoxine restores almost normal hemoglobin levels of the

patients, this new protease seems to play certain roles in the development of this anemia. Therefore, investigation into the regulatory mechanisms of ALA synthetase levels by this new protease in erythroblasts of patients with pyridoxine-responsive anemia is considered important to clarify the pathogenesis of this disease.

In this paper we determined ALA synthetase activity in erythroblasts of two patients with pyridoxine-responsive anemia during the course of the treatment with pyridoxal phosphate. The new protease activity in erythroblasts of the patients was also measured. Furthermore, apo-ALA synthetase was prepared from erythroblasts of the patients to examine both the apparent affinity to pyridoxal phosphate and the susceptibility to the new protease in mitochondria of erythroblasts.

METHODS

Patients. Two cases with pyridoxine-responsive anemia of classical type were employed. Clinical features of the patients are summarized in Table I. Before treatment they showed hypochromic, microcytic anemia with severe poikilocytosis, elevated serum iron with high saturation of total iron binding capacity, erythroid hyperplasia in the bone marrow, and the presence of a large number of ring sideroblasts. They responded to the administration of pharmacological doses of pyridoxal phosphate (60–210 mg/d, orally) with reticulocytosis, and the anemia was gradually improved. Nearly normal hemoglobin levels were maintained by the treatment.

Assay of ALA synthetase activity. ALA synthetase activity was measured by the method of Aoki et al. (9) after partial purification of the enzyme from aspirated bone marrow cells. Cellular components, which were obtained by centrifuging the bone marrow aspirate (5×10^6 – 5×10^7 of erythroblasts), were hemolyzed with 4 vol of water for 10 min. After restoring isotonicity by adding 11.5% KCl solution they were centrifuged at 10,000 g for 10 min. The precipitate that was washed with 1.15% KCl containing 0.01 M potassium phosphate buffer (pH 7.2) was suspended in 10 ml of the same buffer. It was sonicated for 1 min in ice water (20 kcyc/s, 120 W, model UR-150P, Tomy, Tokyo), and then centrifuged at 20,000 g for 20 min. From the precipitate ALA synthetase

TABLE I
Clinical Features of the Patients

| Patients | Age | Sex | Hb | Total eryth $\times 10^4$ | Hct | WBC | Pl $\times 10^4$ | Serum iron | TIBC |
|------------------|-----|------|----------|------------------------------|-----|-----------------------|---------------------|----------------|------|
| | | | g/100 ml | cells/mm ³ | % | cells/mm ³ | | μ g/100 ml | |
| Case No. 1 | 20 | male | | | | | | | |
| Before treatment | | | 2.1 | 85 | 6.5 | 8,100 | 38.0 | 245 | 300 |
| After treatment | | | 13.5 | 412 | 40 | 4,500 | 25.7 | 106 | 326 |
| Case No. 2 | 11 | male | | | | | | | |
| Before treatment | | | 5.0 | 162 | 14 | 7,900 | 13.0 | 245 | 268 |
| After treatment | | | 13.6 | 452 | 40 | 7,600 | 12.1 | 108 | 316 |

Abbreviations used in this table: eryth, erythrocytes; Hb, hemoglobin; Hct, hematocrit; Pl, platelets; TIBC, total iron binding capacity; WBC, leukocytes.

was extracted with 1.5–2.0 ml of 0.2% sodium deoxycholate as described (9). Usually 70–80% of the enzyme activity was recovered in the extracted supernate (9). The enzyme activity was measured using [14 C]succinyl-CoA as the precursor, and incubating at 37°C for 30 min. [14 C]ALA formed was isolated by Dowex (Dow Corning Corp., Midland, Mich.) 50 W \times 8 column chromatography to be counted by a liquid scintillation counter as described (9). In this procedure ALA formation as a function of incubation time was linear up to 60 min, and [14 C]ALA formed was recovered above 95% in the eluate from Dowex column (9). 1 U was defined as the amount of the enzyme activity that produces 1 nmol of ALA in 30 min under the conditions described (9).

Cell separation. Cell separation of bone marrow cells was performed as described (10). Bone marrow aspirate (3–4 ml) diluted with 3 vol of saline was centrifuged at 650 g for 10 min to obtain cellular components. The precipitate suspended in 20 ml of saline was filtered through four layers of gauze. The filtrate was layered on a separating medium consisting of two parts (each part: 10 ml), which were made by mixing appropriate amount of Lymphoprep (Nyegaad & Co. AS, Oslo, Norway) and saline. Specific gravity of each part was 1.067 (upper part) and 1.073 (lower part). After centrifugation at 170 g for 30 min the cell layers formed between the sample and the upper part, and between the upper and lower part were collected to obtain the erythroblast-rich fraction, which contained chiefly erythroblasts (20–50%) and lymphocytes (40–70%), and the immature granulocyte-rich fraction, which contained mainly immature granulocytes (85–90%), respectively. Each fraction was washed three times in saline by centrifugation before the determination of the protease activity.

Preparation and assay of the new serine protease. Since the new serine protease activity was detected in mitochondria of both erythroblasts and granulocytes, it was purified and further crystallized from human bone marrow cells obtained from resected human ribs (10). The protease, which resembles but is not identical with elastase, inactivated specifically the apoform of certain pyridoxal enzymes. Therefore, the protease activity was measured by the use of apo-ornithine transaminase as substrate as described (10). Hematological cells (5×10^6 – 1×10^7) suspended in 3 ml of 0.85% NaCl containing 0.01 M potassium phosphate buffer (pH 7.5) were sonicated at 50 W for 15 s in a 50-ml centrifuge tube (Branson Sonic Power Co. Danbury, Conn., model W 185). From the precipitate obtained by centrifuging the sonicated solution at 20,000 g for 15 min the protease was extracted with 1 ml of 0.5 M potassium phosphate buffer (pH 7.0) for 30 min at 37°C. 1 U was defined as the amount of protease inactivating 50% of apo-ornithine transaminase under the conditions described (10). When the tube containing the protease activity below 2 U was employed, the semilogarithmic degradation curve of apo-ornithine transaminase was linear up to 30 min. However, it became nonlinear in 10 min when the tube containing the activity above 6 U was employed. Therefore, for the determination of the protease activity, tubes containing the protease activity below 6 U were used and the reaction was stopped at 10 min.

Preparation of apo-ALA synthetase. Since the ALA synthetase activity in both granulocytes and lymphocytes is very low (10) as compared with that in erythroblasts (below 1:200), the enzyme activity in aspirated bone marrow cells is considered to indicate the activity in erythroblasts. Therefore, ALA synthetase for the preparation of the apo-enzyme was obtained from the sonicated precipitate of the whole bone marrow aspirate (2×10^7 – 6×10^7 of erythroblasts) by the extraction with 1.5–2.0 ml of 0.2% sodium deoxycholate as described above. It was applied on Sephadex G-25

(Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) column (1 \times 15 cm) equilibrated with 0.2 M Tris (pH 7.0) containing 0.1 mM hydroxylamine. The protein fractions were collected and dialyzed against 0.2 M Tris (pH 7.0) containing 0.1 mM hydroxylamine for various hours at 4°C. They were then subjected to CM-cellulose column (1 \times 15 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 7.5) to obtain apo-ALA synthetase, which appeared at the void volume.

Inactivation of apo-ALA synthetase by the protease. 0.3 ml of the apo-ALA synthetase solution containing the enzyme activity of 0.28–0.40 U was used as the substrate for the protease. Incubation mixture contained apo-ALA synthetase solution, 0.3 ml; dithiothreitol, 0.5 μ mol; potassium chloride, 50 μ mol; potassium phosphate buffer (pH 8.5), 50 μ mol; and various amount of the protease in a final volume of 0.5 ml. After incubation at 37°C for 10 min the reaction was terminated by cooling in ice, and 50 μ g of elastinal (11) was added to each tube. The remaining apo-ALA synthetase was converted to the holoform by incubating the reaction mixture with pyridoxal phosphate (0.2 mM) at 37°C for 10 min, and the ALA synthetase activity was measured. The protease activity in the preparation of apo-ALA synthetase was also measured by the method described above. In each experiment examining the susceptibility of apo-ALA synthetase to the protease three different amounts of the protease were added to the incubation mixture, and the degree of inactivation was plotted against the amount of the protease in the incubation mixture. Susceptibility of apo-ALA synthetase to the protease was expressed as the units of susceptibility. 1 U was defined as the degree of inactivation losing one-half of the activity in 30 min under the conditions described above.

Measurement of K_m of apo-ALA synthetase to pyridoxal phosphate. For the determination of K_m of apo-ALA synthetase to pyridoxal phosphate 0.3 ml of apo-ALA synthetase solution containing 0.28–0.40 U of the activity was employed. The reaction mixture containing apo-ALA synthetase solution, 0.3 ml; dithiothreitol, 0.5 μ mol; potassium chloride, 100 μ mol; elastinal, 50 μ g; and various amounts of pyridoxal phosphate in a final volume of 0.5 ml was incubated at 37°C for 30 min. ALA synthetase activity was then measured without further addition of pyridoxal phosphate. The activity of holo-ALA synthetase contained in the preparation of apo-ALA synthetase (about 30% of the activity was usually holoenzyme in the preparation of apo-ALA synthetase) was subtracted from each value to obtain the net ALA synthetase activity formed from the apo-ALA synthetase. Reciprocals of the ALA synthetase activity vs. pyridoxal phosphate concentration in the incubation mixture were plotted to estimate K_m of the enzyme to pyridoxal phosphate.

RESULTS

Changes of ALA synthetase activity during the course of treatment with pyridoxal phosphate. Fig. 1 shows the changes of ALA synthetase activity in erythroblasts of the patient (case No. 2) during the treatment with pyridoxal phosphate. Before therapy ALA synthetase activity was markedly decreased as shown in this figure. He responded to pyridoxal phosphate (210 mg/d, orally) with reticulocytosis. 4 wk after the beginning of the treatment anemia was markedly improved. At this time bone marrow aspiration was performed to measure ALA synthetase activity in erythroblasts. Approximately fivefold increment of the activity was observed. His anemia was almost com-

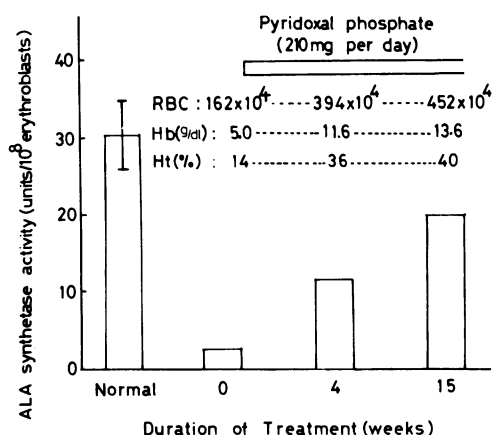


FIGURE 1 Changes of the ALA synthetase activity during the course of treatment with pyridoxal phosphate. The patient (case No. 2) was treated with pyridoxal phosphate (210 mg/d, orally), and ALA synthetase activity in erythroblasts was measured before treatment, 4 wk, and 15 wk after the beginning of the treatment. Hematological data at the time of examination were also shown in the figure. Bars, SD ($n = 19$).

pletely improved 15 wk after the beginning of the treatment. The ALA synthetase activity at this time was increased to nearly normal level. The enzyme activity in erythroblasts of case No. 1 was also extremely decreased before treatment (1.1 U/10⁸ erythroblasts). However, the activity was markedly increased (18.5 U/10⁸ erythroblasts) when anemia was improved by the administration of pyridoxal phosphate (90 mg/d, orally).

ALA synthetase activity measured without addition of pyridoxal phosphate. For the estimation of the amount of apo-ALA synthetase in erythroblasts of the patients before treatment, ALA synthetase activity was measured without addition of pyridoxal phosphate to the incubation mixture, and it was compared with the enzyme activity measured in the presence of pyridoxal phosphate. Even a large amount of pyridoxal phosphate (0.1 mM) failed to enhance the enzyme activity of both patients.

The new serine protease activity in erythroblasts of patients with pyridoxine-responsive anemia. Because the erythroblast-rich fraction prepared as described above contained a small number of immature granulocytes (5–15%) besides both erythroblasts and lymphocytes, it was necessary to estimate the protease activity by the calculations as follows. The immature granulocyte-rich fraction contained mainly immature granulocytes (85–90% of immature granulocytes and 5–10% of lymphocytes). The state of maturity of immature granulocytes contained in each fraction was almost the same (mainly myelocytes and metamyelocytes). Lymphocytes were proved to contain negligible protease activity (10), therefore, the protease activity in immature granulocytes was calculated by counting

the percentage of immature granulocytes in a stained blood film of immature granulocyte-rich fraction. Increment of the protease activity measured with addition of immature granulocytes (up to 30%) to the erythroblast-rich fraction was almost the same as the activity contained in the immature granulocytes. Therefore, the protease activity in erythroblasts was calculated by counting the percentage of both erythroblasts and immature granulocytes in a stained blood film of the erythroblast-rich fraction. As shown in Table II the protease activity in erythroblasts of patients with pyridoxine-responsive anemia was within normal range.

Conversion of ALA synthetase to its apoform. ALA synthetase obtained was converted to its apoform by dialysis against 0.2 M Tris (pH 7.0) containing 0.1 mM hydroxylamine. Fig. 2 shows the degree of conversion to the apoform of the ALA synthetase in erythroblasts by the dialysis for various hours. 2 h dialysis of the enzyme from normal controls resulted in 60–70% conversion to its apoform. 75–85% that of normal controls was converted to its apoform by the dialysis for 6 and 12 h, respectively. Inactivation of the enzyme from normal controls during the procedure for conversion to its apoform was only slight even when dialysed for 12 h as shown in Fig. 2. Degree of conversion to the apoform and inactivation of the enzyme during the procedure for the conversion were also examined using the enzyme solution obtained from the patients. Although degree of conversion to its apoform by 2 h dialysis was similar to that of normal controls (70% of the enzyme was converted to its apoform), ALA synthetase in erythroblasts of the patients was markedly inactivated during the dialysis for 12 h at 4°C as shown in Fig. 2. This marked inactivation is strikingly different from that of normal controls. On the other hand, the experiment shown in Fig. 2, employing the ALA synthetase from case No. 2, indicates that the inactivation was almost completely prevented by elastatinal, the inhibitor to this protease.

Inactivation of apo-ALA synthetase by the protease. Apo-ALA synthetase solutions (protein concentration: 0.05–0.07%) obtained by the dialysis for 2 h were employed as the substrate for the protease, because

TABLE II
Protease Activity in Erythroblasts of Patients with Pyridoxine-responsive Anemia

| Cases | Protease activity |
|------------------------------|---------------------------------|
| | U/10 ⁸ erythroblasts |
| Normal controls ($n = 16$) | 6,825 ± 1,023 (mean ± SD) |
| Case No. 1 | 7,820 |
| Case No. 2 | 7,200 |

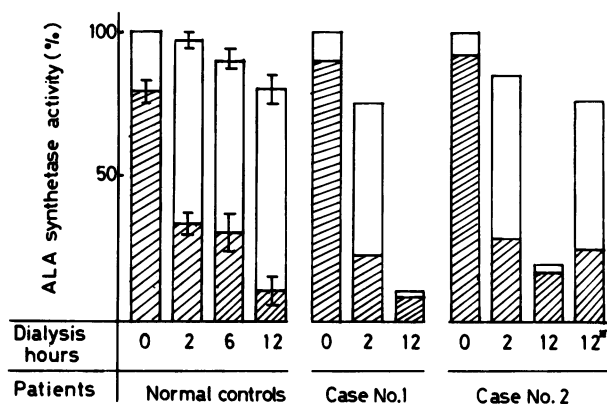


FIGURE 2 Changes of the ALA synthetase activity and conversion of the enzyme to its apoform during dialysis. Deoxycholate extracts containing ALA synthetase from erythroblasts were subjected to Sephadex G-25 column equilibrated with 0.2 M Tris (pH 7.0) containing 0.1 mM hydroxylamine, and the protein fractions (protein concentration: 0.05–0.07%) were dialyzed against 0.2 M Tris (pH 7.0) containing 0.1 mM hydroxylamine for various hours at 4°C. In the experiments shown in this figure comparing the degrees of both inactivation and conversion to apoenzyme during dialysis the apoenzyme obtained was not subjected to CM-cellulose column to avoid various degrees of dilution. The enzyme activity was measured after dialysis against three changes of 100 vol of 0.05 M potassium phosphate buffer (pH 7.5) for 2 h without addition of pyridoxal phosphate (holo-ALA synthetase activity), and with addition of 0.1 mM pyridoxal phosphate (holo- plus apo-ALA synthetase activity) to the incubation mixture. Columns indicate the enzyme activity measured with addition of pyridoxal phosphate. The hatched area of the column shows the enzyme activity measured without addition of pyridoxal phosphate (holo-ALA synthetase activity). The enzyme activity was expressed as the percentage of that of zero-hour dialysis. The enzyme solutions from the patients were obtained when they were in remission. Bars, SD ($n = 6$). *The enzyme solution was prepared by dialysis for 12 h against 0.2 M Tris (pH 7.0) containing both hydroxylamine 0.1 mM and elastatinal (100 μ g/ml). It was further dialyzed against three changes of 100 vol of 0.05 M potassium phosphate buffer (pH 7.5) containing elastatinal (100 μ g/ml) for 2 h before the measurement of the enzyme activity.

conversion to the apoform was equally sufficient for the enzyme of both normal controls and pyridoxine-responsive anemia, and because prolonged dialysis inactivated the enzyme from the patients as shown in Fig. 2. Degrees of inactivation of apo-ALA synthetase by various amounts of protease are shown in Fig. 3. Inactivation curves as a function of the amount of protease of apo-ALA synthetase from 15 normal controls and from various diseases except pyridoxine-responsive anemia were rather in narrow range. Addition to the apo-ALA synthetase solutions of the material (up to 50% of total protein), which was obtained from the normal peripheral blood by the same procedure for the preparation of apo-ALA synthetase, did not change the degree of inactivation of apo-ALA synthetase by the protease. Also the protease activity measured by the

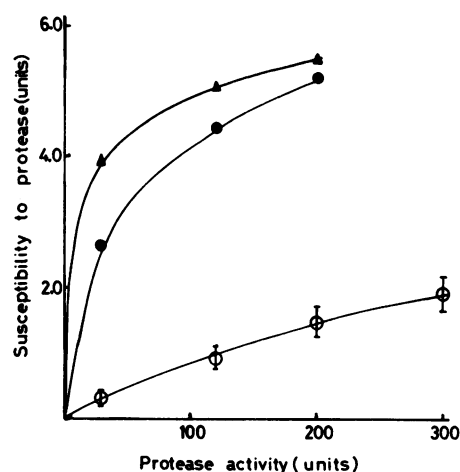


FIGURE 3 Susceptibility of apo-ALA synthetase to protease. Apo-ALA synthetase solutions were incubated at 37°C for 10 min in the presence of various amounts of protease to measure the degree of inactivation by protease as described in Methods. 1 U of the susceptibility to protease was defined as the degree of inactivation losing one-half of the enzyme activity in 30 min under the conditions described in Methods. The mean \pm SD of 15 normal controls (○), case No. 1 (▲), and case No. 2 (●).

inactivation of apo-ornithine transaminase was only additive when various amounts of purified protease (30–400 U) were added to 0.3 ml of apo-ALA synthetase solutions. Apo-ALA synthetase obtained from erythroblasts of patients with iron deficiency anemia, stomach cancer, uterus cancer, rheumatoid arthritis, and primary acquired sideroblastic anemia showed the similar inactivation curves as those of normal controls. On the other hand, apo-ALA synthetase obtained from erythroblasts of patients with pyridoxine-responsive anemia was easily inactivated by the addition of a small amount of the protease as shown in Fig. 3.

Effect of elastatinal on the inactivation of apo-ALA synthetase by the protease. This new serine protease is known to be inhibited by a small amount of elastatinal ($ID_{50} = 3.6 \mu$ g/ml) as described (10). Elastatinal, which was obtained from the cultured broth of *Actinomyces*, was already shown to inhibit specifically the enzyme activity of both elastase from pancreas (11) and this new protease (10). Inactivation by this protease of apo-ALA synthetase from both normal controls and pyridoxine-responsive anemia was completely inhibited by the addition of elastatinal as shown in Fig. 4.

Inactivation of holo-ALA synthetase by the protease. For the comparison of the extent of inactivation by the protease between holo- and apoform of the enzyme, apo-ALA synthetase obtained was first converted to the holoform by incubating with pyridoxal phosphate, and then incubated with the protease. Degree of inactivation of holo-ALA synthetase by the protease was compared with that of the apoenzyme. As shown in Fig.

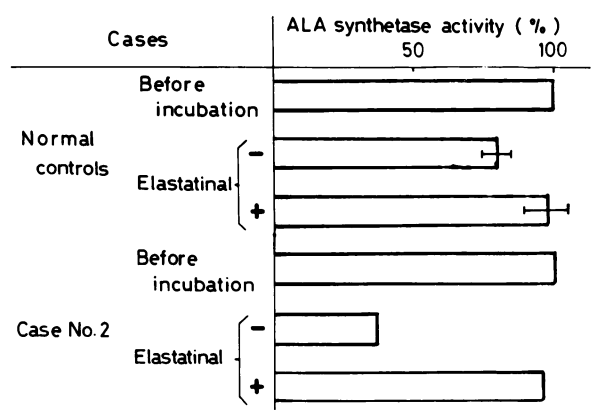


FIGURE 4 Effect of elastatinal on the inactivation of apo-ALA synthetase by protease. Elastatinal, the inhibitor to this protease, was added to the incubation mixture (100 μ g/ml), and then inactivation of apo-ALA synthetase by protease (120 U in each tube) was measured as described in Methods. Inactivation of apo-ALA synthetase by protease in the absence of elastatinal was also shown in the figure. Apo-ALA synthetase solutions from erythroblasts of the patient were obtained when he was in remission by the treatment with pyridoxal phosphate. The activity was expressed as the percentage of that of tubes not incubated with protease. Bars, SD ($n = 5$).

5 inactivation by the protease of holo-ALA synthetase obtained from both normal controls and pyridoxine-responsive anemia was negligible as compared with that of the apo-ALA synthetase.

Apparent affinity (K_m) of apo-ALA synthetase to pyridoxal phosphate. For the determination of the affinity of apo-ALA synthetase (K_m) to pyridoxal phos-

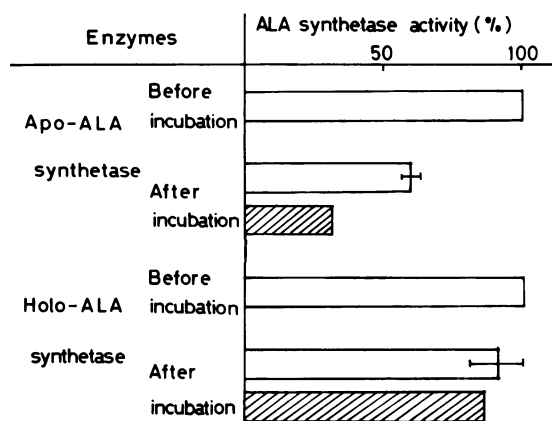


FIGURE 5 Inactivation of holo-ALA synthetase by protease. Preparations of apo-ALA synthetase were incubated with pyridoxal phosphate (0.2 mM) at 37°C for 10 min to obtain the holoenzyme, and the inactivation by protease (300 U in each tube) was measured as described in Methods. Inactivation of apo-ALA synthetase by protease (300 U in each tube) was also shown in the figures. Hatched columns show the results of experiments employing the enzyme from case No. 2 when he was in remission.

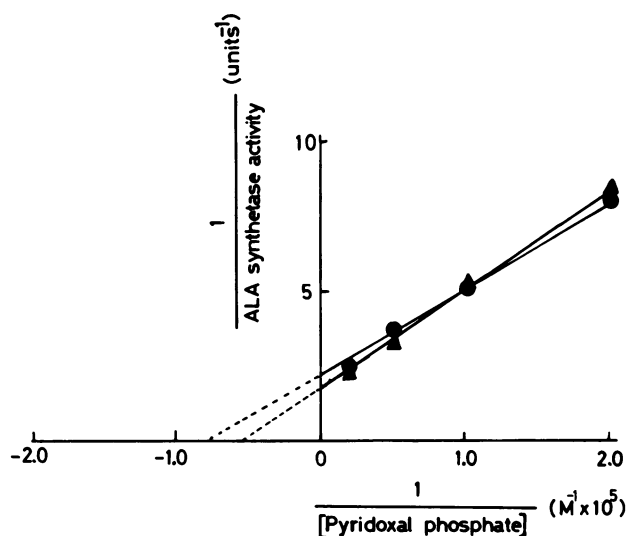


FIGURE 6 Double-reciprocal relationship between ALA synthetase activity and pyridoxal phosphate concentration. Apo-ALA synthetase solutions from the patients were pre-incubated with various amounts of pyridoxal phosphate at 37°C for 30 min, and then the enzyme activity was measured without further addition of pyridoxal phosphate as described in Methods. The double-reciprocal relationship between the enzyme activity and pyridoxal phosphate concentration was plotted. Case No. 1 (\blacktriangle); case No. 2., (\bullet).

phate apo-ALA synthetase was preincubated with various amounts of pyridoxal phosphate as described in Methods. The amount of holoenzyme converted from apoenzyme was then determined by measuring the enzyme activity without further addition of pyridoxal phosphate. Double-reciprocal relationship between the enzyme activity vs. the concentration of pyridoxal phosphate was plotted as shown in Fig. 6. The relationship gave straight lines. From this figure the concentration of pyridoxal phosphate required for the half saturation of the existing apo-ALA synthetase (K_m to pyridoxal phosphate) was obtained. Apo-ALA synthetase obtained from seven normal controls showed the K_m of $0.5\text{--}1.5 \text{ M}^{-1} \times 10^5$. As shown in Fig. 6 each K_m of the enzyme obtained from erythroblasts of two patients with pyridoxine-responsive anemia was similar to that of normal controls.

DISCUSSION

Almost all features of pyridoxine-responsive anemia could be ascribed to the disturbance of heme synthesis in erythroblasts (9, 12–15). The major defect in heme synthetic pathway of erythroblasts of patients with primary sideroblastic anemia including those with pyridoxine-responsive anemia is considered to lie at the step of ALA formation from succinyl-CoA and glycine, which is catalyzed by ALA synthetase, the rate-limiting enzyme in heme synthetic pathway

(8, 9, 16). Among enzymes in heme synthetic pathway only ALA synthetase requires pyridoxal phosphate as a cofactor. Therefore, the remarkable response to the administration of pyridoxine of the patients with this disease is speculated to be through the effect on the ALA synthetase in erythroblasts. ALA synthetase activity in erythroblasts of two cases reported here was extremely decreased before treatment. Parallel increment was observed between the ALA synthetase activity in erythroblasts and hemoglobin levels in the peripheral blood when the patients were treated with pyridoxal phosphate as shown in Results. Therefore, it is considered that reduced hemoglobin synthesis as well as decreased erythrocyte production due to the ineffective erythropoiesis is caused by the decrease of ALA synthetase activity in erythroblasts, and that improvement of hemoglobin synthesis as well as increase of erythrocyte production in the bone marrow by the treatment with pyridoxal phosphate is brought about through the increase of ALA synthetase activity in erythroblasts.

Three mechanisms are considered as the reason for the increment of ALA synthetase activity in erythroblasts of patients with pyridoxine-responsive anemia by the treatment with pyridoxine: (a) increased formation of the holoenzyme from the existing apoenzyme by a large amount of pyridoxal phosphate; (b) induction of apo-ALA synthetase by pyridoxal phosphate; and (c) prevention of degradation of the enzyme by the cofactor. The result that addition of a large amount of pyridoxal phosphate to the partially purified ALA synthetase from erythroblasts of the patients before treatment failed to enhance the enzyme activity shows that amount of apo-ALA synthetase was also extremely decreased in erythroblasts of patients with pyridoxine-responsive anemia before treatment. Furthermore, the fact that apo-ALA synthetase obtained from erythroblasts of both patients showed the same K_m to pyridoxal phosphate as that of normal controls indicates that the decreased ALA synthetase activity in erythroblasts and its restoration by the administration of a large amount of pyridoxal phosphate of the patients with pyridoxine-responsive anemia are not a result of the decreased affinity of the apoenzyme to pyridoxal phosphate. Papers that reported enzyme induction by pyridoxal phosphate (17) are rather scarce. On the contrary, pyridoxal phosphate is considered to increase enzyme levels through the stabilization of the apoenzymes, but not through induction of enzymes (18, 19). Therefore, in pyridoxine-responsive anemia the possibility also remains that ALA synthetase in erythroblasts degrades more rapidly than that of normal persons, and that degradation of the enzyme is prevented by the administration of a large amount of pyridoxine.

Recently, we found a new protease that is considered

to be engaged in the regulation of ALA synthetase levels in erythroblasts by degrading the apoform of the enzyme. The result that the protease activity in erythroblasts of both patients was within normal range shows that the decrease of ALA synthetase activity is not caused by the elevation of the protease activity in erythroblasts. On the other hand, the marked inactivation of ALA synthetase from the patients as compared with that from normal controls during the procedure for the conversion to the apoform, and also the prevention of inactivation of the enzyme obtained from one patient by elastatinal indicate that apo-ALA synthetase in erythroblasts of the patients may possess an increased susceptibility to this protease (90–140 U of the protease activity were usually contained in the preparation of 0.3 ml of apo-ALA synthetase obtained by dialysis). As shown in Results apo-ALA synthetase obtained from the patients was proved to be extremely susceptible to this protease as compared with that from normal controls. Increased susceptibility to the protease of apo-ALA synthetase of the patients may be a result of certain conformational alterations of the apoenzyme, but purification of the enzyme is necessary to discuss the nature of the enzyme. This abnormality of the enzyme may arise from a somatic mutation or the defect may be present at birth but become apparent when the protease activity in erythroblasts increases to the normal level (the activity in bone marrow cells of rats was very low at birth and increased gradually thereafter). Because ALA synthetase levels in erythroblasts are considered to be regulated through degradation of the apoenzyme by this protease, almost all features characteristic to pyridoxine-responsive anemia could be ascribed to the hypercatabolism of ALA synthetase in erythroblasts caused by the increased susceptibility of apo-ALA synthetase to this protease. Pharmacological doses of pyridoxine are considered to be necessary to maintain nearly normal hemoglobin levels, because a large amount of pyridoxal phosphate is required to keep a large holo- to apoenzyme ratio to prevent the degradation of the enzyme.

Although the decreased affinity of apoenzymes to their cofactors is generally considered as the pathogenesis of various vitamin dependency syndromes (20–24), the same mechanism as that of pyridoxine-responsive anemia may also be clarified by detailed examinations in other vitamin dependency syndromes. This is the first instance of the disease that is considered to be caused by the hypercatabolism of an enzyme as a result of its increased susceptibility to an intracellular protease.

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