Control of Pyrimidine Biosynthesis in Human Lymphocytes

SIMULTANEOUS INCREASE IN ACTIVITIES OF GLUTAMINE-UTILIZING CARBAMYL PHOSPHATE SYNTHETASE AND ASPARTATE TRANSCARbamylASE IN PHYTOHEMAGGLUTININ-STIMULATED HUMAN PERIPHERAL LYMPHOCYTES AND THEIR ENZYME CO-PURIFICATION

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SUMMARY

Human peripheral lymphocytes were incubated in the presence of Phaseolus vulgaris phytohemagglutinin. Activities of both glutamine-utilizing carbamyl phosphate synthetase (EC 2.7.2.1) and aspartate transcarbamylase (EC 2.1.3.2), the initial enzymes of the de novo pathway for pyrimidine biosynthesis, increased simultaneously during the blastogenesis of lymphocytes. A very close connection between these two enzyme activities was recognized, the ratio of their activities at various times of culture being nearly constant. The elevation of the two enzyme activities could be prevented either by actinomycin D or puromycin. They were co-purified from phytohemagglutinin-stimulated lymphocytes through ammonium sulfate fractionation, hydroxypatite chromatography, sucrose gradient centrifugation, and zone electrophoresis in sucrose density gradient. The molecular weight of the enzyme complex was estimated to be approximately 600,000. The results indicate that the glutamine-utilizing carbamyl-P synthetase and aspartate transcarbamylase are induced together as an associated form during the blastogenesis of lymphocytes by phytohemagglutinin.

EXPERIMENTAL PROCEDURE

Materials—L-[U-14C]Aspartic acid was purchased from the Radiochemical Centre, Amersham, England. Dilithium carbamyl-P was synthesized according to the method of Jones et al. (12) and further purified by the method of Gerhart and Pardee (13). Thirty S [14C]lysine-labeled ribosomal subunit and 16 S and 23 S ribosomal RNAs prepared from Escherichia coli Q13 (14, 15) were gifts from Dr. Syozo Osawa, Hiroshima University. The sources of the other isotopes and chemical reagents used in this study were as described (16).

Preparation and Culture of Lymphocytes—The procedures were as described (16).

Purification of the Enzymes—Procedures for the preparation of sonicated extracts of lymphocytes were as described previously (16). The purification of carbamyl-P synthetase was performed by the methods used for the preparation of Yoshida ascites hepatoma AH 130 carbamyl-P synthetase (17).

Enzyme Assay—Carbamyl-P synthetase activity was determined as described previously (16). One unit of carbamyl-P synthetase is defined as that amount of enzyme which produces 1 pmole of carbamyl-P per min at 37°C when L-glutamine is used as the nitrogen donor. The isolation of L-[14C]carbamylaspartate produced in this assay was carried out by the method of Porter et al. (18) or Procedure 2 of Bresnick et al. (19). One unit of aspartate transcarbamylase is defined as that amount of enzyme which produces 1 pmole of carbamyl-P per min at 37°C when L-glutamine is used as the nitrogen donor. The isolation of L-[14C]carbamylaspartate produced in this assay was carried out by the method of Porter et al. (18) or by Procedure 2 of Bresnick et al. (19). One unit of aspartate transcarbamylase is defined as that amount of enzyme which produces 1 pmole of carbamylaspartate per min at 37°C.

Radioactivity and Chemical Determination—The determinations of radioactivity, protein, and citrulline were as described previously (16). The determination of carbamylaspartic acid

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was carried out by a modification (13) of the method of Koritz and Cohen (20).

**Paper Electrophoresis of Carbamylaspartic Acid—**Electrophoresis was performed at 5.0 volts per cm for 2 1/2 hours on a Toyo Roshi No. 53 paper with 0.6 m pyridine acetate buffer (pH 6.8) (21). Carbamylaspartic acid was located by direct visualization on paper by spraying with 0.04% phenol red dissolved in ethyl alcohol which was adjusted slightly alkaline with NaOH. Aspartate was stained with ninhydrin as described (9).

**Sucrose Gradient Centrifugation—**To determine an approximate S value of the enzyme, two kinds of standard markers, L-[14C]lysine-labeled 30 S ribosomal subunit and 16 S plus 23 S ribosomal RNA were centrifuged in separate tubes as follows. A sucrose gradient from 5 to 20% containing 20 mm Tris-Cl (pH 7.3), 3 mm 2-mercaptoethanol, 1 mm dithiothreitol, 1 mm EDTA, 50 mm KCl, 4.5 mm MgCl₂, 3 mm ATP, 4 mm L-glutamine, 5% (v/v) glycerol, and 30% (v/v) dimethylsulfoxide was prepared for the enzyme. Reagents involved as enzyme stabilizers, 2-mercaptoethanol, dithiothreitol, EDTA, ATP, and L-glutamine, were omitted from sucrose gradients for the standard markers since no significant increase in viscosity of the sucrose gradient was expected by the addition of these reagents. A sucrose gradient for 30 S ribosomal subunit contained 10 mm Tris-Cl (pH 7.3), 0.1 mm MgCl₂, 5% (v/v) glycerol, 30% (v/v) dimethylsulfoxide, and sucrose. A sucrose gradient for 16 S and 23 S ribosomal RNAs contained 10 mm Tris-Cl (pH 7.3), 0.1 mm MgCl₂, 50 mm KCl, 5% (v/v) glycerol, 30% (v/v) dimethylsulfoxide, and sucrose. The enzyme preparation or the standard markers (each 0.5 ml) was layered on each sucrose gradient (15 ml) and centrifuged together in a SW 25.3 rotor of a Beckman model L2 ultracentrifuge at 25,000 rpm at 2°C for 66 hours. After centrifugation, fractionation was carried out by the method of Oumi and Osawa (22), followed by the radioactivity determination for 30 S ribosomal subunit and by ultraviolet absorption at 260 nm for 16 S and 23 S ribosomal RNAs.

**Zone Electrophoresis in Sucrose Density Gradient—**The apparatus and procedures for zone electrophoresis in sucrose density gradient have been described (23). Buffer solutions used in this experiment contained 50 mm Tris-Cl, 3 mm 2-mercaptoethanol, 1 mm dithiothreitol, 1 mm EDTA, 50 mm KCl, 4.5 mm MgCl₂, 3 mm ATP, 4 mm L-glutamine, 5% (v/v) glycerol, 30% (v/v) dimethylsulfoxide, and sucrose. Each solution was finally adjusted to pH 7.8. The sample dissolved in 0.5 ml of the buffer solution, adjusted in density to 2.5%, with respect to sucrose, was layered on the top of the sucrose column, 20 ml of linear sucrose gradient from 20% to 5%. The apparatus was kept in water at 0°C. After the electrophoretic run for 12 hours under constant current of 10 ma, drops from the sampling needle were collected into 0.45-ml fractions.

**RESULTS**

**Simultaneous Increase in Activities of Carbamyl-P Synthetase and Aspartate Transcarbamylase—**Lymphocytes were incubated in the presence of phytohemagglutinin. Activities of carbamyl-P synthetase and aspartate transcarbamylase of sonicated extracts of lymphocytes at various periods after the addition of phytohemagglutinin were determined (Fig. 1). The radioactive product of the aspartate transcarbamylase reaction which was recovered from the chromatogram of Dowex 50 as described under "Experimental Procedure" was identified as carbamylaspartic acid by its behavior on paper electrophoresis. After an initial lag of about 12 hours, the specific activities of the two enzymes increased simultaneously. A very close correlation of the two curves was recognized, the ratio of the two enzyme activities at 12, 24, 36, 48, and 70 hours of culture being 83, 83, 79, 80, and 80, respectively. The addition of either actinomycin D or puromycin to the cultures after incubation of the lymphocytes with phytohemagglutinin for 36 hours resulted in no further or only slight increase in these enzyme activities, supporting that the two enzymes are induced together during the blastogenesis of lymphocytes.

**Sucrose Gradient Centrifugation—**The enzyme preparation in the step of ammonium sulfate fractionation was subjected to sucrose gradient centrifugation. The activities of carbamyl-P synthetase and aspartate transcarbamylase were recovered in the same fraction (Fig. 2). An approximate S value of the enzyme complex was estimated to be 21 S from its sedimentation position relative to 30 S ribosomal subunit, 17 S from 16 S ribosomal RNA, and 18 S from 23 S ribosomal RNA. This value corresponds to the molecular weight of around 600,000.

**Zone Electrophoresis—**Although electrophoresis on starch or Pevikon C-870 was not successful in the study of electrophoretic behavior of carbamyl-P synthetase in the presence of glycerol and dimethylsulfoxide required to stabilize the enzyme, the enzyme moved slowly without significant inactivation in zone electrophoresis in sucrose density gradient in the presence of the stabilizers. The enzyme preparation in the step of ammonium sulfate fractionation was subjected to electrophoresis. The two activities of carbamyl-P synthetase and aspartate transcarbamylase were found in the same fraction (Fig. 3).
The results in the present paper show that the two enzyme activities of carbamyl-P synthetase and aspartate transcarbamylase, the first and second enzymes of the de novo pathway for pyrimidine biosynthesis, increase concomitantly in phytohemagglutinin-stimulated human lymphocytes and that the two enzymes have been co-purified through ammonium sulfate fractionation containing 108 units of carbamyl-P synthetase and standard markers, 30 S L-[14C]lysine-labeled ribosomal subunit and 16 S ribosomal RNA (560,000) ; 23 S ribosomal RNA (1,100,000) (15). Details of the electrophoresis procedure are described under "Experimental Procedure." Either enzyme activity of carbamyl-P synthetase (○) and aspartate transcarbamylase (△) was determined with 0.05 ml of the enzyme preparation of each fraction. The peaks of the standard markers are indicated by the arrows. Sedimentation direction is from right to left. The molecular weight of the markers is as follows: 30 S ribosomal subunit (700,000) (14); 16 S ribosomal RNA (560,000); 23 S ribosomal RNA (1,100,000) (15).

The results presented here evidently support the conclusion that both enzymes are present as an associated form.

**DISCUSSION**

The results in the present paper show that the two enzyme activities of carbamyl-P synthetase and aspartate transcarbamylase, the first and second enzymes of the de novo pathway for pyrimidine biosynthesis, increase concomitantly in phytohemagglutinin-stimulated human lymphocytes and that the two enzymes have been co-purified through ammonium sulfate fractionation, hydroxylapatite chromatography, sucrose gradient centrifugation, and zone electrophoresis. The data indicate that the two enzymes are co-induced by phytohemagglutinin and the two induced enzymes are present as an associated form.

An approximate molecular weight of the associated form is 600,000. This value corresponds to that of the mouse spleen enzyme reported by Hoogenraad *et al.* (11). The level of activity of aspartate transcarbamylase in human lymphocytes is about 100 times that of carbamyl-P synthetase. A more extensive purification of the two enzymes by procedures such as DEAE-cellulose chromatography or gel filtration chromatography with agarose in the presence of 5% (v/v) glycerol and 30% (v/v) dimethylsulfoxide has not been successful.

While a single carbamyl-P synthetase provides carbamyl-P for the biosynthesis of both pyrimidine and arginine in bacteria (24), two types of carbamyl P synthetase, one for pyrimidine biosynthesis and the other for arginine biosynthesis, are present in fungi (1, 2), amphibians (25), and mammals (9). Details of genetic and biological studies of the pyrimidine-specific carbamyl-P synthetase and aspartate transcarbamylase in *Neurospora* and yeast were reported. In these fungi, the two enzymes are coded by the same genetic region (1, 3, 6) and controlled by co-repression or co-derepression (1, 4). The two enzymes are present as a single complex (5, 6) which serves to channel the product, carbamyl-P, of the first enzyme into the pyrimidine pathway (7, 8). Similari ties in properties of the human lymphocyte and fungus enzymes, co-induction or co-derepression and co-purification of the two enzymes, suggest that the human lymphocyte enzymes correspond to the *Neurospora* and yeast enzymes reported by Hoogenraad *et al.* (11).
enzymes in biological and genetic functions. An operation of the channeling of carbamyl-P into the pyrimidine pathway in human lymphocytes could utilize carbamyl-P for pyrimidine biosynthesis very effectively by minimizing its destruction or loss, since carbamyl-P is very unstable at 37°C (26) and the biosynthesis of carbamyl-P is a limiting step in the over-all pathway (16).

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