TWO TYPES OF CARBAMYL PHOSPHATE SYNTHETASE IN RAT LIVER: CHROMATOGRAPHIC RESOLUTION AND IMMUNOLOGICAL DISTINCTION[1]

Shigetada Nakanishi[2], Kazuhiko Ito[3] and Masamiti Tatibana[4]

Department of Medical Chemistry
Kyoto University Faculty of Medicine
Kyoto, Japan

Received October 23, 1968

The formation of carbamyl-P is the initial step for the biosynthesis of arginine and pyrimidines. Livers of various ureotelic animals are known to contain high activity of a carbamyl-P synthetase which utilizes ammonia but not glutamine as a nitrogen donor and requires N-acetyl-L-glutamate as essential activator (Cohen, 1962). On the other hand, it has been demonstrated recently that rapidly proliferating non-ureotelic tissues, such as hematopoietic mouse spleen (Tatibana and Ito, 1967a), rat fetal liver (Hager and Jones, 1967a), and Ehrlich ascites carcinoma (Hager and Jones, 1967b), contain a different type of carbamyl-P synthetase. The

1/ This study has been supported in part by research grants from the U. S. Public Health Service (TW 00166) and the Scientific Research Fund of the Ministry of Education of Japan.

2/ Recipient of a Sigma Chemical Postgraduate Fellowship.

3/ Present address: Department of Internal Medicine, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Hiroshima, Japan.

4/ Present address: Department of Biochemistry, Chiba University School of Medicine, Chiba, Japan.

The abbreviations used: carbamyl-P, carbamyl phosphate; DMSO, dimethylsulfoxide; Na-EDTA, sodium ethylenediaminetetraacetate.

newly detected enzymes utilize either L-glutamine or ammonia as the nitrogen donor, have no requirement for acetylglutamate, and are thought to provide carbamyl-P for pyrimidine biosynthesis in these tissues. These observations suggest that the glutamine-utilizing enzyme may exist, along with the ammonia-dependent enzyme, in mammalian adult liver, particularly in regenerating liver which is not only ureotelic but also proliferating. Although in past studies (Cohen, 1962) most of the ammonia-dependent enzyme has been recovered in the mitochondrial fraction, some activity has invariably appeared in the supernatant fluid and has then hampered a clear demonstration of the glutamine-utilizing enzyme supposed to occur mainly in the soluble fraction.

This communication deals with the presence of the glutamine-utilizing carbamyl-P synthetase in the high-speed supernatant of homogenates of normal and regenerating rat liver. The enzyme was separated from the ammonia-acetylglutamate-dependent carbamyl-P synthetase on a hydroxylapatite column and was partially characterized. Immunological evidence is presented that these two enzymes are discrete proteins.

Methods and Materials - The procedures for enzyme purification and assay were similar to those developed for the glutamine-utilizing carbamyl-P synthetase from hematopoietic mouse spleen (Tatibana et al., in preparation), and are modifications of the methods described previously (Tatibana and Ito, 1967a). In place of glycerol alone, a mixture of DMSO and glycerol is employed as enzyme stabilizer. Evaluation of the effects of these solvents as a stabilizer will be published elsewhere. The standard assay system contained the following constituents in a final volume of 0.6 ml: 30 µmoles of potassium N-2-hydroxyethyl-piperazine-N'-ethanesulfonate buffer, pH 7.0; 6 µmoles of ATP; 9 µmoles of

$MgCl_2$; 0.3 µmole of L-ornithine; 18 µmoles of KCl; 10 µg of purified ornithine transcarbamylase* from beef or frog liver; enzyme to be assayed; 0.045 ml of DMSO; 7.5 mg of glycerol; 0.075 µmole of Na-EDTA; 10 µmoles of $KH^{14}CO_3$ (5×10^6 c.p.m.); 2 µmoles of L-glutamine or 6 µmoles of ammonium acetate as nitrogen donor; and, where indicated, 3 µmoles of N-acetyl-L-glutamate. The incubation was for 15 minutes at 37°. Subsequent procedures were essentially as described previously (Tatibana and Ito, 1967a).

The ammonia-dependent carbamyl-P synthetase from mammalian liver is so unstable that a homogeneous preparation is not available at present. Therefore, the same type of carbamyl-P synthetase was purified from frog liver (Marshall et al., 1958), and rabbit antisera against the enzyme, which can cross-react with the enzyme from rat liver, were prepared as described by Marshall et al. (1961). The antibody obtained by us formed a precipitin line with the rat liver enzyme, when examined by the agar diffusion technique (Ouchterlony, 1949) and by the immunoelectrophoresis technique (Graber and Williams, 1955). γ_2-Globulin fractions of the antisera were prepared by the procedure of Nichol and Deutsch (1948).

Three female Wistar strain rats were subjected to partial hepatectomy. Regenerating liver, removed 22 hours later, was homogenized with 5 volumes of ice-cold 30% (w/v) glycerol solution containing 0.05 M potassium phosphate, pH 7.0, 10 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM dithiothreitol, and 1.44 mM L-glutamine. The homogenate was centrifuged for 40 minutes at 105,000 g and the supernatant was fractionated with ammonium sulfate (0% - 39%). The pre-

* This amount of ornithine transcarbamylase was sufficient for the conversion of 40 µmoles of carbamyl-P into L-citrulline in 1 minute under optimal conditions.

cipitate was chromatographed on a hydroxylapatite column. Stepwise elution was carried out with increasing concentrations of potassium phosphate from 0.03 M to 0.25 M in a 30% (v/v) DMSO solution containing 5% (w/v) glycerol, 0.1 M KCl, 1 mM dithiothreitol and 0.5 mM Na-EDTA. The solution had a final pH of 7.6 (Elution A). Elution was continued with a different solvent system which contained 10% (w/v) glycerol, in place of 30% DMSO and 5% glycerol, and had a final pH of 6.8. Phosphate concentrations ranged from 0.10 M to 0.20 M (Elution B). In spite of the lower salt concentration, the latter system had a stronger eluting power than the former. Eluted enzyme was concentrated through the use of a small column of hydroxylapatite.

Results and Discussion - As shown in Fig. 1, carbamyl-P synthetase was eluted from the hydroxylapatite column in two peaks (Peak I and Peak II). Peak I was identified as the glutamine-utilizing carbamyl-P synthetase and Peak II as the ammonia-acetylglutamate-dependent enzyme. The enzyme in Peak I was indistinguishable from carbamyl-P synthetase isolated from hematopoietic mouse spleen (Tatibana and Ito, 1967a) with regard to substrate specificities, requirements for activators, pH optima, and sensitivities to pyrimidine nucleotides, particularly UTP.

When normal rat liver was subjected to the same fractionation procedure, a similar chromatographic pattern was obtained. Some variation in the enzyme levels during liver regeneration was observed which offers an interesting problem for further investigation.

It is known that DMSO can change protein conformation as well as the catalytic properties of certain enzymes (Hamaguchi, 1964; Rammler, 1967). This raised the possibility that the appearance

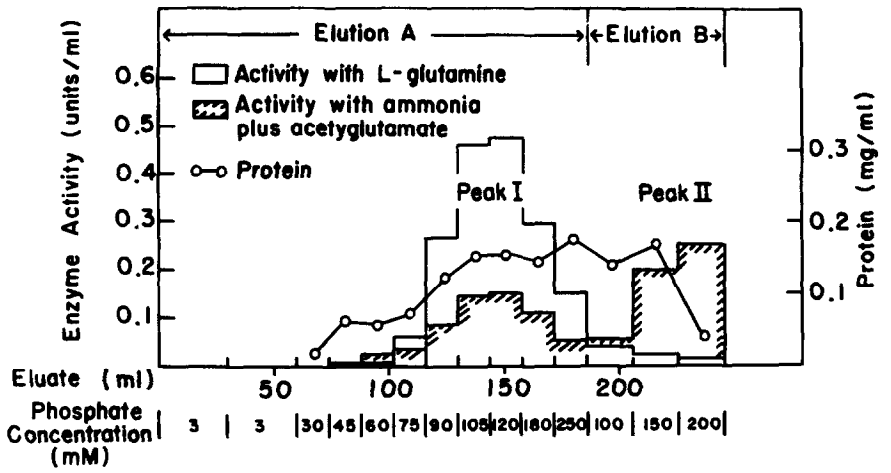

Figure 1. Separation of two types of carbamyl-P synthetase on a hydroxylapatite column with a stepwise gradient of potassium phosphate. For details see text. Enzyme activities were followed in two different assay systems, with 3.3 mM L-glutamine as sole nitrogen donor ☐ and with 10 mM ammonium acetate plus 5 mM N-acetyl-L-glutamate ▨. Inorganic phosphate, contained in the eluates, somewhat inhibited the enzyme activity but the values illustrated here were not corrected. Protein (o——o) was assayed by the method of Lowry et al. (1951). One unit of carbamyl-P synthetase is defined as the amount of enzyme which synthesizes 1 mμmole of carbamyl-P in 1 minute under the assay conditions.

of the two activities was related to an effect of DMSO. Therefore, the following experiment was undertaken. Mitochondria of normal rat liver, prepared by the method of Hogeboom (1955), were disrupted by sonication and centrifuged. The extract, which contained a high activity of the ammonia-dependent enzyme, was subjected to chromatography on a hydroxylapatite column. All buffers used here contained 30% DMSO (v/v) and 5% glycerol (w/v). Only a single peak of carbamyl-P synthetase appeared and it was identified as the ammonia-acetylglutamate-dependent enzyme. No activity of the glutamine-utilizing enzyme was detected. These observations indicate that the glutamine-utilizing enzyme is not an artifact derived from

the ammonia-dependent enzyme during the purification. It was also shown that the glutamine-utilizing enzyme is not present in the mitochondrial fraction.

The immunological relationship of the enzyme proteins of the two types of carbamyl-P synthetase was studied and the results are summarized in Fig. 2. γ_2-Globulin, isolated from rabbit antisera

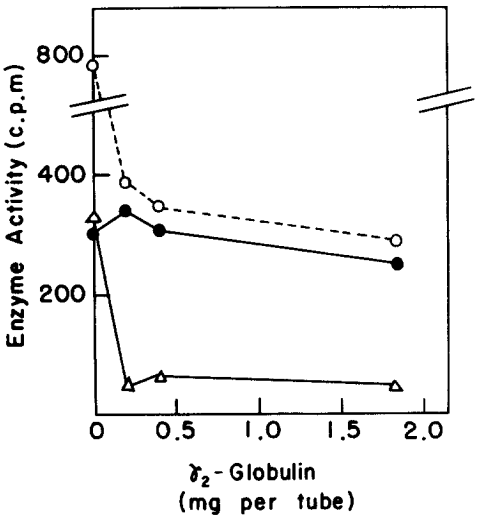

Figure 2. Immunological distinction of two types of carbamyl-P synthetase from rat liver. γ_2-Globulin was isolated from rabbit antisera against ammonia-dependent carbamyl-P synthetase of frog liver. Varying amounts of γ_2-globulin were incubated for 5 minutes at 37° with ammonia-dependent enzyme, 0.5 µg protein (Δ——Δ), with glutamine-utilizing enzyme, 80 µg protein (●——●), or with the mixture of two types of carbamyl-P synthetase (o----o) in a volume of 0.15 ml of 0.13 M Tris-HCl buffer, (pH 7.5) containing 10% DMSO (v/v), 1.7% glycerol (w/v), 33 mM KCl and 100 mM NaCl. The tubes were then kept at 0° for 30 minutes and the whole mixture of each tube was assayed for carbamyl-P synthetase activity. The final concentrations of the reaction components in the assay system were adjusted as close to those in the standard assay mixture as possible. As nitrogen donor, the mixture of L-glutamine (2 µmoles) and ammonium acetate (6 µmoles) plus N-acetyl-L-glutamate (3 µmoles) was used for all tubes.

against ammonia-dependent carbamyl-P synthetase from frog liver, inhibited by more than 80% the same type of enzyme from rat liver, even at a high dilution of the enzyme and in the presence of DMSO.

On the other hand, the antibody did not affect the activity of the glutamine-utilizing carbamyl-P synthetase. In addition, when a mixture of the two types of enzyme was titrated with the antibody, the remaining activity was essentially equal to the sum of the activities obtained by individual titrations. γ_2-Globulin from non-immunized rabbit sera did not affect either enzyme under the same conditions.

The results presented above indicate that the glutamine-utilizing carbamyl-P synthetase exists as a separate entity in rat liver, together with the ammonia-acetylglutamate-dependent enzyme. In view of wide distribution of the glutamine-utilizing enzyme in proliferating tissues which are devoid of arginine synthesizing activity (Tatibana et al., 1967b; Tatibana and Ito, in preparation), it may be assumed that in rat liver the glutamine-utilizing enzyme functions primarily for pyrimidine synthesis, while the ammonia-dependent enzyme serves for arginine synthesis. Distinctions in subcellular localization of the two enzymes lend additional support to the hypothesis. A possibility remains, however, that the two enzymes are localized in distinct cell groups, one in parenchymatous cells and the other in interstitial cells.

Acknowledgment - The authors wish to thank Professor Osamu Hayaishi for his constant interest and encouragement during the course of this work.

References

Cohen, P. P., in "The Enzyme" (Boyer, P. D., Lardy, H., and Myrbäck, K., eds.) Vol. 6, p. 477. Academic Press. New York and London (1962).
Graber, P., and Williams, C. A. Jr., Biochim. Biophys. Acta, 17, 67 (1955).
Hager, S. E., and Jones, M. E., J. Biol. Chem., 242, 5674 (1967a).
Hager, S. E., and Jones, M. E., J. Biol. Chem., 242, 5667 (1967b).
Hamaguchi, K., J. Biochem. (Japan), 56, 441 (1964).

Hogeboom, G. H., in "Methods in Enzymology" (Colowick, S. P., and Kaplan, N. O., eds.) Vol. I, p. 16. Academic Press. New York (1955).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
Marshall, M., and Metzenberg, R. L., and Cohen, P. P., J. Biol. Chem., 233, 102 (1958).
Marshall, M., and Cohen, P. P., J. Biol. Chem., 236, 718 (1961).
Nichol, J. C., and Deutsch, H. F., J. Amer. Chem. Soc., 70, 80 (1948).
Ouchterlony, O., Arkiv. Kemi., B26, 1 (1949).
Rammler, D. H., Ann. N. Y. Acad. Sci., 141, 291 (1967).
Tatibana, M., and Ito, K., Biochem. Biophys. Res. Commun., 26, 221 (1967a).
Tatibana, M., Ito, K., Terada, M., and Inagaki, A., 7th Inter. Congr. Biochem. (Tokyo), G-17, p. 841 (1967b).