AN *IN VITRO* INCREASE IN ASPARTYLTRANSCARBAMYLASE ACTIVITY*

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A number of investigators have demonstrated synthesis of specific enzymes in cell-free systems (cf. Nisman and Pelmont, 1964). The following communication deals with an increase of aspartyltranscarbamylase (ATCase) activity observed in a cell-free system from a uracil requiring mutant of *E. coli*. When grown on limiting amounts of uracil, ATCase contitutes about 12 per cent of the soluble protein of this mutant (Gerhart and Schachman, 1965). The *in vitro* increase in ATCase activity reported here is dependent on the presence of ATP, GTP and Mg^{++} and a dialyzed cell-free enzyme preparation of the mutant. The increase in activity is greater if exogenous amino acids are added and is partially inhibited by RNase, but not by puromycin.

EXPERIMENTAL

The mutant was grown aerobically at 37^{o} to early log phase in a medium containing limiting amounts of uracil (8 μg/ml)[1]. The cells were chilled and all subsequent operations performed at 0 to 5^{o}. The harvested cells were washed in a standard buffer containing 0.01 M potassium phosphate[2] pH 7.4,

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[1] I thank Dr. J. C. Gerhart for providing the mutant of *E. coli* and communicating unpublished observations regarding suitable growth conditions.

[2] Use of phosphate buffer during preparation of the "enzyme" and in the incubation medium more than doubled the increase in ATCase activity above that observed with tris(hydroxymethyl)aminomethane buffer at the same pH.

0.014 M magnesium acetate and 0.06 M KCl, suspended in buffer and disrupted by passage through a French Press at 16,000 psi. The broken cell suspension was centrifuged at 10,000 x g for 30 minutes, the resultant pellet discarded, and the supernatant fluid centrifuged at 30,000 x g for 30 minutes. The supernatant fluid was dialyzed against 100 volumes of standard buffer for 8 hours with a change of buffer after 4 hours. The dialyzed preparation, designated as, "enzyme", was stored in small aliquots at -20° after quick freezing in liquid nitrogen. Once thawed the enzyme was not stored again since thawing and refreezing caused loss of activity. When ribosomes were separated from the enzyme, the concentration of Mg^{++} was raised to 0.03 M and the preparation centrifuged at 105,000 x g for 2 hours. About three fourths of the supernatant fluid was carefully removed by aspiration and used as the 105,000 x g supernatant. The 105,000 x g pellet, was resuspended, sedimented again and used as the ribosome fraction. Both the 105,000 x g supernatant enzyme and ribosomes were stored at -20° after quick freezing in liquid nitrogen.

RESULTS AND DISCUSSION

Table I shows the requirements for an *in vitro* increase in ATCase activity. The increase is dependent on the addition of ATP, GTP and Mg^{++} (experiment A) and to a lesser extent on the addition of amino acids (experiment B). The degree of dependence on exogenous amino acids varies widely with different enzyme preparations from nearly complete dependency to no effect and a typical experiment is shown here. Experiment C shows that both the 105,000 x g supernatent and ribosomes are required for optimal activity.

The increase in ATCase activity and the incorporation of amino acids are inhibited to the same extent (23 and 25 per cent respectively) by 5 µg of RNase (Table II). However, with 100 µg puromycin the increase in activity of ATCase is not inhibited even though incorporation of amino acids into protein is inhibited by about 60 per cent. A concentration of RNase which inhibits incorporation of a mixture of ^{14}C labeled amino acids into protein by only 25 per cent inhibits ^{14}C labeled L-leucine incorporation by more than 90 per cent.

TABLE I

Requirements for an *in vitro* increase in ATCase activity

	milli-units ATCase
Experiment A	
Endogenous ("enzyme" plus buffer)	117
Complete system	240
" " minus ATP	125
" " minus GTP	140
" " minus Mg^{++}	139
Experiment B	
Endogenous ("enzyme" plus buffer)	122
Complete system	227
" " minus amino acids	196
Experiment C	
Supernatant enzyme plus buffer	135
Supernatant enzyme plus incubation mixture	172
Ribosomes plus buffer	18
Ribosomes plus incubation mixture	42
Supernatant plus ribosomes plus buffer	152
Supernatant plus ribosomes plus incubation mixture	267

The complete system contained in μmoles: potassium phosphate 50 (pH 7.4); magnesium acetate 60; ATP 10; GTP 10; (all experimentally determined as being the optimal concentrations) phosphoenolpyruvate 5; 50 μgrams of each of the 20 amino acids and in experiments A and B about 1 mg of enzyme protein (Lowry *et al.*, 1951) in a final volume of 1 ml. In experiment C 105,000 x g supernatant enzyme or ribosomes or the 105,000 x g supernatant plus ribosomes were incubated in buffer alone or in the complete reaction mixture. Incubation was for 10 minutes at 37° and the reaction was terminated by chilling the tubes in an ice bath. From each tube 0.5 ml of the incubated reaction mixture was removed and dialyzed for 3 hours against 100 volumes of 0.01 M potassium phosphate buffer pH 7.4 containing 0.06 M KCl with two changes of the buffer. The dialyzed incubation mixtures were then diluted to 5 ml with the above buffer and an aliquot assayed for ATCase by the method of Gerhart and Pardee (1962). One milli-unit of ATCase represents 1 mμmole of carbamylaspartate synthesized per minute under the conditions of the assay. Each experiment was repeated at least twice and assays run in duplicate. The duplicates rarely varied by more than 10 per cent.

TABLE II

Effect of RNase and puromycin on the *in vitro* increase of ATCase and on the incorporation of a mixture of ^{14}C amino acids and ^{14}C leucine into protein.

	increase in ATCase	amino acids incorporated into protein	^{14}C L-leucine incorporated into protein
	milli-units	mμg	μμmoles
Complete system	56	57	56
" " plus 5 μg RNase	43	42	3.3
" " plus 100 μg puromycin	56	22	3.5

Conditions as in Table I except that 5 μg of each of the 20 amino acids were used. For studies of the incorporation of labeled amino acids a mixture of 5 μg each of fifteen ^{14}C amino acids ("Reconstituted protein hydrolysate" from Schwarz Bioresearch Inc., Orangeburg, N.Y.) and ^{14}C histidine were added to give 9.6×10^5 cpm per tube. Five μg each of unlabeled aspargine, cysteine, cystine, glutamine and methionine were also added. For the studies of incorporation of labeled leucine, 5 μg of ^{14}C L-leucine (specific activity 9 mc/mM) and 5 μg each of the other 19 unlabeled amino acids were added. In studies of inhibition of ATCase increase, 3 to 4 replicates were run as well as controls containing equivalent amounts of RNase or puromycin to rule out any possible inhibition of ATCase activity by these compounds. Control tubes for amino acids or leucine incorporation were incubated at 0°. To measure the incorporation of the label into protein the reaction was terminated by adding 5 ml of 5% trichloroacetate (TCA) and heated for 20 minutes at 90°. The precipitated protein was washed once with 5% TCA, then with 3:1 ethanol:ether and then 5 times on a millipore filter with 5% TCA. Finally the filter containing the washed precipitate was counted with 15 ml of scintillation fluid (10% napthalene, 0.7% 2,5-diphenyloxazole and 0.03% 2,2-p-phenylenebis(5-phenyloxazole) in reagent grade dioxane) in a liquid scintillation spectrometer. Endogenous values for ATCase and the control values (0°) for the incorporation experiments have been subtracted.

The concentration of puromycin which inhibits incorporation of a mixture of amino acids by about 60 per cent also inhibits L-leucine incorporation by more that 90 per cent.

The requirements of the system for an *in vitro* increase in ATCase activity are identical to the requirements for protein synthesizing systems. Therefore,

one is inclined to attribute the observed increase to *de novo* synthesis of ATCase. However, the results obtained with puromycin suggests that this hypothesis is not entirely adequate and a part of the increase must represent other processes. The increase in activity does not seem to be due to the conversion of the oligomeric form of the enzyme to its monomeric form (Gerhart and Schachman, 1965) since treatments known to cause this conversion, such as heating for 4 minutes at 60° or treatment with ATP (Gerhart and Pardee, 1962), do not cause an increase in our system.

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