Arch. Mikrobiol. 73, 97—103 (1970)

Inactivation of Glutamine Synthetase by Adenylylation in Intact Cells of *E. coli*

C. Peter Heinrich and Helmut Holzer

Biochemisches Institut der Universität Freiburg i. Br., Germany
and Forschungsgruppe für Biochemie der Gesellschaft für Strahlenforschung mbH München, Germany

Received April 23, 1970

Summary. The adenine pool of a purineless mutant of *E. coli* was radioactively labelled by short incubation with ^{14}C-adenine.

The glutamine synthetase was inactivated *in vivo* by incubation of the cell suspension with 2×10^{-3} M NH_4^+ for 2 min. The inactivated glutamine synthetase was extracted from the cells and purified 20-fold.

Incubation of the purified glutamine synthetase with phosphodiesterase regenerated the biosynthetic activity of the enzyme paralleled by the liberation of ^{14}C-adenine and ^{14}C-adenosine. ^{14}C-adenine and ^{14}C-adenosine were also obtained when inactivated glutamine synthetase, prepared *in vitro* by use of ^{14}C-ATP and purified adenylylating enzyme, was incubated with phosphodiesterase under the same conditions.

The similar liberation of adenine derivatives by phosphodiesterase from glutamine synthetase inactivated in a cell-free system as well as in intact cells, demonstrates that in both cases the inactivation consists in an adenylylation of the enzyme.

After addition of NH_4^+ ions to a suspension of *E. coli* cells grown without NH_4^+, one observes within 1—2 min an inactivation of glutamine synthetase to a few percent of its original activity (Mecke and Holzer, 1966). In cell-free extracts of *E. coli* glutamine synthetase is rapidly inactivated in the presence of ATP and Mg^{++} by the addition of glutamine (Mecke *et al.*, 1966a). The inactivation in the cell-free system is catalyzed by an enzyme (Mecke *et al.*, 1966b) and is an ATP-dependent adenylylation of glutamine synthetase, stimulated by glutamine according to the following equation (Wulff *et al.*, 1967; Kingdon *et al.*, 1967):

$$\text{Glutamine synthetase} + 12\ \text{ATP} \xrightarrow{\text{(glutamine)}} \text{glutamine synthetase(-AMP)}_{12} + 12\ \text{PP}.$$

In intact cells inactivation of glutamine synthetase is caused by 10^{-4} M NH_4^+. Glutamine is effective only in a 100-fold higher concentration (Holzer *et al.*, 1967). In the cell-free system, however, 10^{-3} M glutamine causes inactivation, while NH_4^+ remains without any effect even at 10^3-fold concentration.

According to these striking differences of the metabolites initiating inactivation in intact cells and in the cell-free system, respectively, one would expect different mechanisms of the inactivation process. There are, however, some indications that the same mechanism is responsible for the two processes (Holzer *et al.*, 1967; Holzer, 1967). In both cases the transferase activity during inactivation of the enzyme is much less affected than the biosynthetic activity (Mecke and Holzer, 1966). Furthermore, among five different enzymes studied, the inactivation is specific for glutamine synthetase (Mecke and Holzer, 1966) in intact cells as well as in the cell-free system. For further elucidation of the problem whether different mechanisms of inactivation could account for the difference of the metabolic effectors, the experiments described in the present paper have been carried out. It will be shown that the inactivation of glutamine synthetase by ATP-dependent adenylylation, already demonstrated in the cell-free system, occurs also in intact cells.

Materials and Methods

Strain and Growth Conditions. A mutant of *E. coli*, *E. coli Br*, was obtained by treatment of the wild type of *E. coli* with N-methyl-N-nitroso-N′-nitroguanidine as mutagen and selection for purine requirement (Eisenstark *et al.*, 1965). As described earlier (Mecke and Holzer, 1966) the bacteria were grown on minimal medium. Optimal concentration of adenine for growth of the mutant was found to be 72 μmoles/l. The bacteria, 2 l of cell suspension, were grown to an optical density of 0.5 (650 nm) and then centrifuged for 10 min at 34000 × g. In order to double the cell density, the centrifuged cells were resuspended in 1 l of medium without nitrogen source and without adenine for 15 min at 37°C. During these 15 min the glutamine synthetase activity increased about 10-fold.

8-^{14}C-Adenine Uptake. Two 500 ml portions of the resuspended cells were incubated with 2 μmoles of 8-^{14}C-adenine (specific activity 25.6 mc/m mole) for 10 min at 37°C. The 8-^{14}C-adenine uptake by the cells was followed by measuring the decrease of radioactivity in the medium as follows: 100 μl cell suspension were added to 0.9 ml ice-cold water and centrifuged immediately at 15000 × g for 2 min. 100 μl of the supernatant were taken for liquid scintillation counting. The scintillation liquid was made up of 800 ml toluene, 400 ml abs. ethanol and 42 ml Liquifluor. Two thirds of the ^{14}C-adenine radioactivity from the medium are taken up by the cells during 8 min of incubation.

Inactivation. After incubation with 8-^{14}C-adenine, the glutamine synthetase in cell suspension No. 1 was inactivated by addition of NH_4^+ up to a concentration of 2×10^{-3} M. Suspensions No. 1 and 2 were then incubated for 2 min at 37°C, cooled in an ice bath, and centrifuged for 10 min at 34000 × g.

Purification of Glutamine Synthetase. The pellets of both charges, 1 g moist weight each, were ground with 3 g Alcoa, and the glutamine synthetase was isolated as described earlier (Mecke and Holzer, 1966).

Reactivation. 200 μl of the purified inactivated glutamine synthetase, 7.5 mM $MgSO_4$, 130 μl 1 M Tris pH 9 and 60 μl snake venom phosphodiesterase (1.0 mg/ml) were incubated at 37°. 50 μl of this incubation mixture were taken at different times (see Fig. 1) for determination of the glutamine synthetase activity, and 20 μl were

used for ^{14}C-assay according to the procedure of Mans and Novelli (Mans and Novelli, 1961). For comparison, non-inactivated glutamine synthetase was treated in the same manner.

Chemicals. 8-^{14}C-adenine was purchased from Radiochemical Centre (Amersham, Buckinghamshire) CFA. 348 Batch 5, spec. act. 51.1 mc/mM),

adenine and ATP from C. F. Boehringer Mannheim GmbH (Mannheim),

Alcoa (A 305 bacteriological grade) from Aluminium Corp. of America (Pittsburgh, Pa.),

Liquiflor from the New England Nuclear Corp. (Boston, Mass.),

streptomycinsulfate from Serva Entwicklungslabor (Heidelberg),

snake venom phosphodiesterase from Worthington Biochemical Corp. (Freehold, New Jersey).

All the other materials used were p.a. substances from Merck AG (Darmstadt).

Electrophoresis. The buffer employed for tank electrophoresis (apparatus from F. Hüttinger [Freiburg]) was pyridine acetate, pH 3.6 (10 ml pyridine, 100 ml acetic acid, 890 ml water). Conditions for electrophoresis: 65 V/cm, 45 mA, 1—3°, Schl. & Sch. paper No. 2043 b Mgl.

100 µl of each of the incubation mixtures, described in section "Reactivation", were taken at zero time and after 4 h, were precipitated by 50 µl 50% trichloroacetic acid and centrifuged. 20 µl supernatant were applied to electrophoresis. The electropherogram was cut into 1×4 cm strips and examined for radioactivity in the liquid scintillation counter.

Thin-Layer Chromatography. Was performed as described by Shapiro *et al.*, 1967.

Results

An inactivation to less than 10% of the control value has occurred in the NH_4^+ treated cells (legend to Fig. 1). Glutamine synthetase was purified about 20-fold from the untreated and NH_4^+-inactivated cell suspension. As shown by Shapiro *et al.*, 1967, snake venom phosphodiesterase can be used in order to split off covalently bound AMP from glutamine synthease. Therefore, if the inactivation goes parallel to an adenylylation, incubation of the inactivated glutamine synthetase with snake venom phosphodiesterase should result in a reactivation parallel to liberation of AMP. To prove this, the purified glutamine synthetases from both cell suspensions were incubated with phosphodiesterase. Fig. 1 (left ordinate) shows the increase of synthetase activity in the case of inactivated glutamine synthetase, whereas the synthetase activity of the non-inactivated glutamine synthetase remains fairly unchanged.

Parallel to the reactivation with phosphodiesterase, radioactive AMP should be liberated from adenylylated glutamine synthetase. Fig. 1 (right ordinate) demonstrates the changes of ^{14}C-radioactivity in the trichloroacetic acid-insoluble material as measured with the procedure of Mans and Novelli (Mans and Novelli, 1961). There is a decrease in the case of inactivated glutamine synthetase, but no decrease in the case of non-inactivated glutamine synthetase.

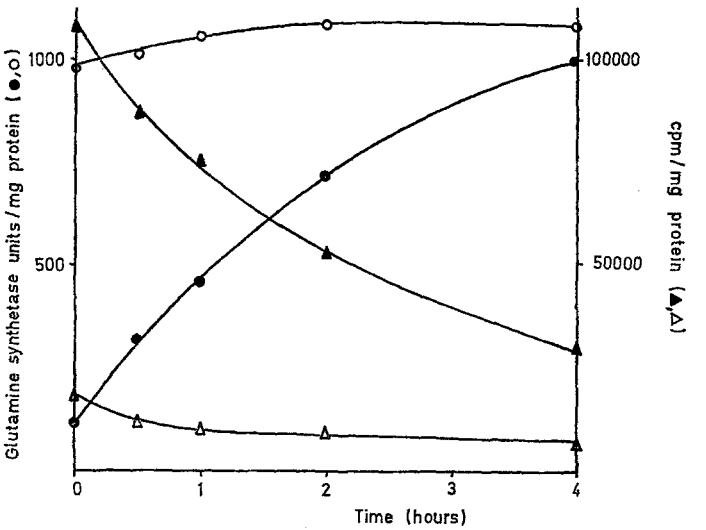

Fig. 1. *Incubation of glutamine synthetase preparations with snake venom phosphodiesterase.* After streptomycin precipitation, 2 acid precipitations and 60° treatment, a specific biosynthetic activity of glutamine synthetase of 123 was obtained from the NH_4^+-inactivated cell suspension (1 g moist cells). The specific activity of glutamine synthetase isolated from the cell suspension, where no NH_4^+ was added, was 1390. *Left ordinate:* synthetase activity of the incubation mixture with non-inactivated glutamine synthetase (open circles); synthetase activity of the incubation mixture with inactivated glutamine synthetase (black dots). *Right ordinate:* radioactivity of the trichloro-acetic acid-insoluble material of the incubation mixture with non-inactivated glutamine synthetase (open triangles); radioactivity of the trichloro-acetic acid-insoluble material of the incubation mixture with inactivated glutamine synthetase (black triangles)

The increase of biosynthetic activity of the glutamine synthetase preparation from NH_4^+-inactivated cells should correspond to the decrease of protein-bound radioactivity. The plot of synthetase activity/mg protein versus cpm/mg protein for the different times during incubation with phosphodiesterase shows a fairly good linear dependency.

In order to make sure that the decrease of ^{14}C-radioactivity in the trichloro-acetic acid-insoluble material is indeed caused by the release of AMP, efforts were made to identify the ^{14}C-labelled material liberated. For this purpose, unlabelled AMP was added to aliquots of the incubation mixtures of the glutamine synthetases with snake venom phosphodiesterase. The reaction was stopped by addition of trichloro-acetic acid. After high voltage paper electrophoresis, it was possible to detect the authentic AMP which had moved towards the anode, but no radioactivity was found on the AMP spot. However, as shown in Fig. 2, two new

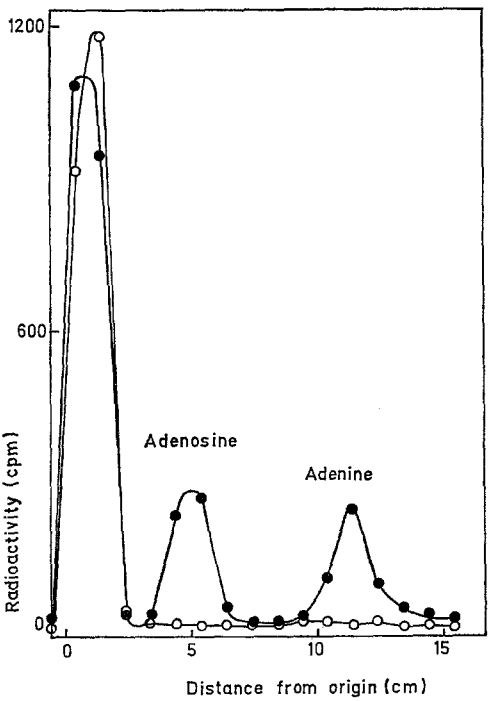

Fig. 2. *Electrophoresis of the trichloro-acetic acid supernatant from NH_4^+ inactivated ^{14}C-labelled glutamine synthetase preparations before (open circles) and after 4 h (black dots) incubation with phosphodiesterase.* The high radioactivity peak close to the origin is attributed to trichloro-acetic acid soluble material (see Fig. 1, right ordinate) which contaminates both glutamine synthetase preparations

peaks of ^{14}C-radioactivity appeared on the cathodic part of the electropherogram. The electrophoretic mobilities of the two peaks correspond exactly to those of authentic adenosine and adenine. For further identification, the electropherogram was eluted at the positions of radioactivity and, after lyophilisation, the radioactive materials were applied to thin-layer cellulose plates. The cellulose layer was scratched off in form of 1×4 cm strips and added directly to the scintillation liquid. The total radioactivity was detected at the same positions as unlabelled authentic adenosine and adenine.

For comparison with these results from glutamine synthetase inactivated in intact cells, *in vitro* inactivated and ^{14}C-labelled glutamine synthetase was incubated under identical reaction conditions with phosphodiesterase. Again, no ^{14}C-AMP could be found, but ^{14}C-adenosine and ^{14}C-adenine were detected.

Discussion

In earlier experiments, it was shown *in vitro* by incorporation of ^{14}C-radioactivity from ^{14}C-labelled ATP into the enzyme-protein that the inactivation of glutamine synthetase is an ATP-dependent adenylylation (Wulff *et al.*, 1967; Kindgon *et al.*, 1967). In the present paper the inactivation of glutamine synthetase was studied in intact cells. The ATP pool was ^{14}C-labelled by brief incubation of a purineless mutant of *E. coli* with ^{14}C-adenine. After inactivation of glutamine synthetase in intact cells by NH_4^+-addition, the enzyme was purified from the cell extracts 20-fold. The purified enzyme preparation has the same properties as glutamine synthetase obtained by inactivation with ^{14}C-ATP *in vitro*, i.e., the ester-bond of the adenylic acid to the protein is split by incubation with phosphodiesterase and the biosynthetic activity is regenerated parallel to the liberation of ^{14}C-adenine containing material. Instead of the expected ^{14}C-AMP, ^{14}C-adenine and ^{14}C-adenosine were formed. This is attributed to the hydrolytic action of enzymes contaminating the diesterase preparation. These results demonstrate that the inactivation of the biosynthetic activity of glutamine synthetase caused by NH_4^+-addition to intact cells of *E. coli* has the same chemical mechanism as it was found with pure enzymes, i. e., an ATP-dependent, enzyme-catalyzed inactivation of glutamine synthetase by adenylylation.

In the cell-free system the adenylylating enzyme is effective only after addition of a stimulating effector. NH_4^+ is without effect, glutamine is a potent effector, certain other amino acids are also effective (Mecke and Holzer, 1966a; Wulff *et al.*, 1967). It is probable that in intact cells NH_4^+ reacts rapidly with glutamate to yield glutamine, which stimulates the adenylylating enzyme (Holzer, 1969).

Acknowledgements. The work was supported by the Bundesministerium für Bildung und Wissenschaft. We are indebted to Drs. A. Holldorf and D. Mecke for useful comments. The mutant of *E. coli* was a generous gift of Dr. A. Holldorf.

References

Eisenstark, A., Eisenstark, R., Van Sickle, R.: Mutation of *Salmonella typhimurium* by nitrosoguanidine. Mutation Res. **2**, 1—10 (1965).

Holzer, H.: Inactivation et activation enzymatiques, contrôlées par un métabolite de la glutamine synthétase de *E. coli*. Bull. Soc. Chim. biol. (Paris) **49**, 1421—1430 (1967).

— Regulation of enzymes by enzyme-catalyzed chemical modification. Advanc. Enzymol. **32**, 297—326 (1969).

— Mecke, D., Wulff, K., Liess, K., Heilmeyer, L.: Metabolite-induced enzymatic inactivation of glutamine synthetase in *E. coli*. In: Advances in Enzyme Regulation (G. Weber, ed.), Vol. 5, pp. 211—225. Oxford-New York: Pergamon Press Ltd. 1967.

Kingdon, H. S., Shapiro, B. M., Stadtman, E. R.: Regulation of glutamine synthetase, VIII. ATP: glutamine synthetase adenylyltransferase, an enzyme that catalyzes alterations in the regulatory properties of glutamine synthetase. Proc. nat. Acad. Sci. (Wash). **58**, 1703—1710 (1967).

Mans, R. J., Novelli, G. D.: Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. Arch. Biochem. **94**, 48—53 (1961).

Mecke, D., Holzer, H.: Repression und Inaktivierung von Glutaminsynthetase in *Escherichia coli* durch NH_4^+. Biochim. biophys. Acta (Amst.) **122**, 341—351 (1966).

— Wulff, K., Holzer, H.: Metabolit-induzierte Inaktivierung von Glutaminsynthetase aus *Escherichia coli* im zellfreien System. Biochim. biophys. Acta (Amst.) **128**, 559—567 (1966a).

— Wulff, K., Liess, K., Holzer, H.: Characterization of a glutamine synthetase inactivating enzyme from *Escherichia coli*. Biochem. biophys. Res. Commun. **24**, 452—458 (1966b).

Shapiro, B. M., Kingdon, H. S., Stadtman, E. R.: Regulation of glutamine synthetase, VII. Adenylyl glutamine synthetase: a new form of the enzyme with altered regulatory and kinetic properties. Proc. nat. Acad. Sci. (Wash.) **58**, 642—649 (1967).

Wulff, K., Mecke, D., Holzer, H.: Mechanism of the enzymatic inactivation of glutamine synthetase from *E. coli*. Biochem. biophys. Res. Commun. **28**, 740—745 (1967).

Prof. Dr. H. Holzer
Biochem. Inst. der Universität
D-7800 Freiburg im Breisgau
Hermann-Herder-Straße 7