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A proteolytic artifact associated with the lysis of bacteria by egg white lysozyme

(protease/esterase/protein degradation)

C. N. OLIVER AND E. R. STADTMAN

Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

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ABSTRACT **Polyacrylamide gel electrophoresis of cell-free extracts of *Escherichia coli* that had been grown in a medium containing $^{32}P_i$ disclosed the presence of several ^{32}P-labeled proteins. Comparison of the electrophoretic patterns obtained in the presence of carrier unlabeled purified *E. coli* glutamine synthetase before and after treatment with trypsin, subtilisin, or snake venom phosphodiesterase showed that most of the ^{32}P was present in the adenylyl moieties of adenylylated glutamine synthetase. Low molecular weight ^{32}P-labeled degradation products of glutamine synthetase were also observed in extracts prepared by treatment of cells with lysozyme but not in extracts prepared by sonic oscillation. The degradation of glutamine synthetase in lysozyme-prepared extracts is likely due to an intrinsic proteolytic activity of egg white lysozyme. Proteolysis probably occurs at the esterase site of lysozyme described by Piszkiewicz and Bruice [Piszkiewicz, D. & Bruice, T. C. (1968) *Biochemistry* 7, 3037–3047]. Selective carboxymethylation of lysozyme histidine-15 leads to simultaneous loss of esterase and protease activities but only to partial loss of lytic activity. In view of these findings, caution is needed in the interpretation of results obtained with extracts of cells prepared by lysozyme treatment, especially when such extracts are used to investigate the properties of proteolytic enzymes.**

The control of intracellular enzyme levels is important in metabolic regulation. Variation in enzyme levels reflects alterations in either the rates of protein synthesis or the rates of degradation. Whereas much is known about the mechanism and regulation of protein synthesis, efforts to understand the mechanism of specific enzyme degradation and the factors that control it have been hampered by inability to obtain cell-free extracts that catalyze the differential degradation of specific enzymes. To determine whether earlier failures might have been due to inactivation of the degrading system by conventional cell disruption procedures, we and others (1) have prepared cell-free extracts by gentle lysis of the cells with lysozyme (mucopeptide *N*-acetylmuramoylhydrolase, EC 3.2.1.17) treatment in the presence of either EDTA (2) or high salt concentrations (3). To facilitate the search for an active degradation system in such extracts, the bacteria were grown in a medium containing $^{32}P_i$ so that the fate of phosphorylated or nucleotidylated proteins could be monitored after the extraction procedure. We report here that [*adenylylate*-^{32}P]glutamine synthetase [L-glutamate: ammonia ligase (ADP-forming), EC 6.3.1.2] is the major ^{32}P-labeled protein in $^{32}P_i$-grown cells and that the labeled enzyme undergoes proteolytic degradation in lysozyme-prepared extracts but not in extracts prepared by sonic oscillation. The proteolytic activity of lysozyme-treated extracts was shown to be an artifact caused by an intrinsic activity of egg white lysozyme and is the manifestation of a nonspecific esterase activity (4) of the enzyme. This serves notice that, in the absence of appropriate controls to discount the lysozyme effect, the physiological significance of studies of protein degradation in extracts prepared with lysozyme is in doubt.

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MATERIALS AND METHODS

Chemicals. Chymotrypsin and trypsin were obtained from Boehringer Mannheim. Casein, albumin, azocasein and azoalbumin, carboxypeptidase A, carboxypeptidase B, protease from *Streptomyces griseus*, benzoyl-L-tyrosine ethyl ester, and *N*-tosyl-L-arginine methyl ester were purchased from Sigma.

Bacterial Strains. *E. coli* strains NF314 (*leu*$^-$, *valS*$^+$, *relA*$^+$), NF536 (*leu*$^-$, *valS*^{ts}, *relA*$^+$), and NF537 (*leu*$^-$, *valS*^{ts}, *relA*$^-$) were provided by Anthony Furano (National Institutes of Health). These strains were used in this study because the initial observations were made with these cells.

Growth of Cells. Cells were grown on Tris/glucose medium adapted from Kaempfer and Magasanik (5); 20 mM glucose was substituted for glycerol. In ^{32}P-labeling experiments, cultures were exposed to $^{32}P_i$ (New England Nuclear; 5–10 μCi/ml of culture fluid; 1 Ci = 37 GBq) for two generations; parallel unlabeled cultures were also prepared. All cultures were grown aerobically with vigorous shaking at 30°C.

Preparation of Cell Extracts. Cells were harvested, washed twice in 50 mM Tris·HCl buffer (pH 7.0) and concentrated to 1/10th vol. Cells were disrupted by lysozyme treatment according to the method of Kaback (2) or, alternatively, by the method of Wickner *et al.* (3); comparable results were obtained with either procedure. The second method eliminated the use of EDTA which promotes subunit dissociation of glutamine synthetase. Cells were also disrupted by sonic oscillation at 4°C with a Heat Systems sonifier cell disrupter (model W185). In all cases, the resulting suspension was clarified by centrifugation at 27,000 × *g* for 20 min.

Electrophoresis. Slab polyacrylamide gradient gels of 4–30% acrylamide with 4% crosslinking were prepared by a method adapted from Margolis and Kendrick (6). The running buffer was 25 mM Tris·HCl/197 mM glycine, pH 8.3. In all experiments, 10–40 μg of protein was loaded per well and electrophoresis was carried out for 2,250 V·hr at 4°C. Similar gradient gels were used for $NaDodSO_4$ electrophoresis except that 0.1% $NaDodSO_4$ was included in the gel buffer and in the running buffer. $NaDodSO_4$ electrophoresis was carried out for 300 V·hr at 25°C. Labeled gels were sliced into 1- to 1.5-mm sections. Each section was incubated in an assay vial with 0.5 ml of NCS tissue solubilizer/water (9:1) (Amersham/Searle) at 52°C for 3 hr. Vials were cooled, 10 ml of Aquasol-2 (New England Nuclear) was added, and the samples were assayed in a Beckman LS-250 liquid scintillation counter.

Abbreviation: CM-lysozyme, carboxymethyl-lysozyme.

Native gels were stained with Coomassie blue G-250 according to the method of Reisner *et al.* (7) and $NaDodSO_4$ gels were stained with 0.1% Coomassie blue R-250 in methanol/water/acetic acid (1:1:0.2) for 2 hr at 30°C. Gels were destained electrophoretically in 10% acetic acid.

Preparation of 3-Carboxymethylhistidine-15 Lysozyme. Specific modification of the histidine-15 residue of lysozyme was carried out according to the method of Piszkiewicz and Bruice (4) and the product was designated CM-lysozyme. Control lysozyme was incubated without iodo[^{14}C]acetic acid (New England Nuclear) and chromatographed under conditions identical to those used with the treated enzyme.

Amino Acid Analysis. Amino acid analysis was carried out using a Dionex amino acid analyzer and calibration data were provided by R. L. Levine.

Enzyme Assays. Nonspecific protease activity was determined using denatured hemoglobin (8), casein, albumin (9), azocasein, and azoalbumin (10). Carboxypeptidase A activity was assayed according to the method of Folk and Schirmer (11), and carboxypeptidase B was determined according to the method of Folk *et al.* (12). Chymotrypsin was assayed using benzoyl-L-tyrosine ethyl ester according to the method of Hummel (13), and trypsin was determined by a similar method using *N*-tosyl-L-arginine methyl ester.

Lysozyme was assayed by the turbimetric method of Shugar (14). Nonspecific esterase activity was determined using *p*-nitrophenylacetate according to the method of Huggins (15) except that 50 mM Tris·HCl buffer (pH 7.5) was used. Glutamine synthetase activity and average state of adenylylation were determined by using the pH 7.57 λ-glutamyltransferase assay (16). [^{14}C]AMP-labeled glutamine synthetase was prepared according to the method of Rhee (details to be published elsewhere).

Protein. Protein was assayed by using the Coomassie blue method of Bradford (17).

Lysozyme. Several different batches of lysozyme were purchased from Worthington, Boehringer Mannheim, and Sigma. All batches of lysozyme were chromatographed on Bio-Rex 70 (Bio-Rad) using a gradient of 50–200 mM potassium phosphate buffer (pH 7.18).

RESULTS

Proteolytic Activity in Lysozyme Preparations. When extracts of ^{32}P-labeled cells were prepared by lysis with lysozyme and EDTA and then subjected to pore gradient electrophoresis, ^{32}P-containing material of high molecular mass (600,000), as well as several discrete highly reproducible bands ranging downward to 30,000 daltons, was observed (Fig. 1). The electrophoretic pattern was not altered by treatment with charcoal, 1% streptomycin sulfate, or with RNAse, DNAse, alkaline phosphatase, or phospholipase (at the arbitrary concentration of 10 μg/mg of extract protein) (data not shown). However, the pattern was obliterated with trypsin, subtilisin (10 μg/mg of extract protein), or snake venom phosphodiesterase (10 μg/mg of extract protein) that had been treated to remove 5′-nucleotidase and protease activities (18). This suggested that the labeled fractions contained proteins esterified with ^{32}P-labeled derivatives. *E. coli* glutamine synthetase is a dodecamer of 12 identical subunits (50,000 daltons each). The activity of the enzyme is modulated *in vivo* and *in vitro* by reversible adenylylation that is regulated by an elaborate enzymic cascade system. Each of the 12 subunits can be modified by covalent attachment of a single adenylyl group and the activity of the enzyme is proportional to the number of unadenylylated subunits. Because electrophoresis of glutamine synthetase in the presence of EDTA is known to yield multiple bands resulting from varying degrees

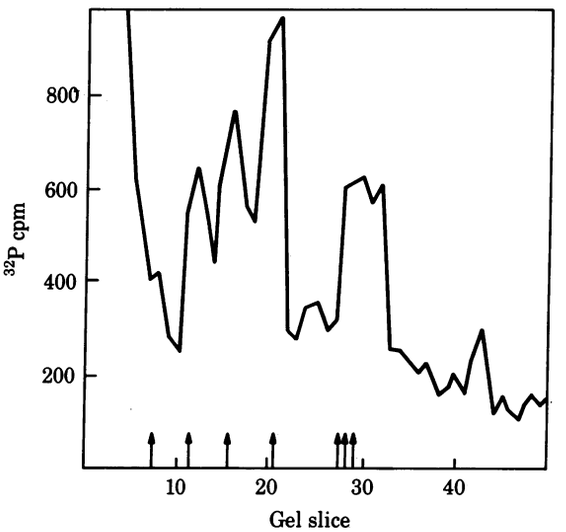

FIG. 1. Native pore gradient electrophoresis of purified unlabeled glutamine synthetase with a ^{32}P-labeled extract of NF536 prepared by lysozyme/EDTA treatment. Arrows indicate positions of Coomassie blue-staining bands that are single and multiple subunit forms of glutamine synthetase. Note the peaks of ^{32}P-labeled protein (fractions 32 and 43) that are smaller than glutamine synthetase subunits (fractions 27–29).

of subunit dissociation (19) and because the adenylyl group can be removed by phosphodiesterase (20), it seemed likely that some of the labeled bands shown in Fig. 1 were derived from ^{32}P-labeled adenylylated glutamine synthetase. This is further supported by the demonstration that the glutamine synthetase in these extracts contained approximately 10 equivalents of adenylyl groups per mol and by the fact that, when labeled extracts were electrophoresed (in the presence of EDTA) together with excess of purified unlabeled glutamine synthetase (to serve as a protein marker), there was a correspondence between the phosphorylated bands and the Coomassie blue-staining bands of ≥50,000 daltons. This correspondence is indicated by the arrows in Fig. 1.

Fig. 1 shows also that, in addition to radioactive fractions of 50,000–600,000 daltons, there are several radioactive fractions of lower molecular mass (e.g., fractions 32 and 43) that do not comigrate with the purified unlabeled enzyme. To determine whether these low molecular mass fractions were derived from glutamine synthetase by the action of proteases in the cell-free extract, purified [^{14}C]adenylylated glutamine synthetase was incubated for 3 hr with lysozyme-prepared extracts of unlabeled cells. To avoid complications that might arise from EDTA-induced dissociation of glutamine synthetase subunits, the extracts used in these experiments were prepared by sonic oscillation or by the modified procedure of Wickner (3) in which EDTA is replaced by high concentrations of salt; multiple small species of ^{14}C-labeled material were generated in lysozyme-prepared extracts (Fig. 2*B*) but not in extracts prepared by sonic oscillation (Fig. 2*C*). Electrophoresis of the purified labeled glutamine synthetase after a 3-hr incubation (Fig. 2*A*) or electrophoresis of a mixture of the labeled enzyme with lysozyme-prepared extracts without incubation (data not shown) revealed no small labeled fragments. Fig. 3 shows that low molecular weight fragments were generated when the same [^{14}C]adenylylated glutamine synthetase was incubated with purified lysozyme. $NaDodSO_4$ electrophoresis of identical incubation mixtures confirmed the presence of labeled fragments smaller than glutamine synthetase subunits. These results indicated that proteolysis of the glutamine synthetase had occurred during

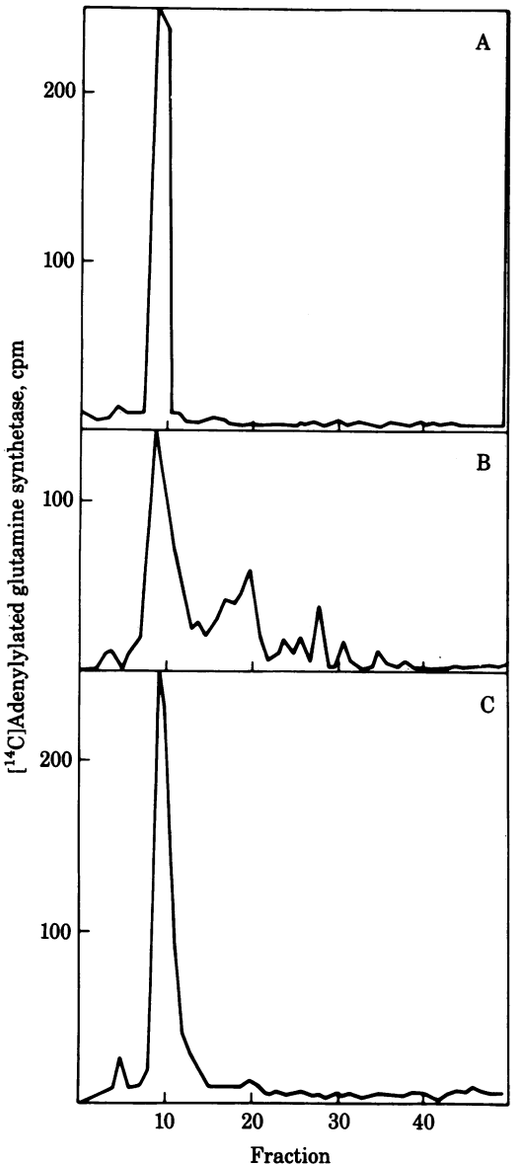

FIG. 2. Native pore gradient electrophoresis of [^{14}C]adenylylated glutamine synthetase after incubation with various extract preparations. Sixteen-microgram samples of [^{14}C]adenylylated glutamine synthetase were incubated alone for 3 hr at 37°C (*A*), with 25 μg of lysozyme-prepared extract (*B*), or with 25 μg of sonicated extract (*C*).

incubation and ruled out the possibility that lysozyme merely facilitated the glutamine synthetase subunit dissociation.

Subsequent experiments using *p*-nitrophenylacetate as a substrate revealed the presence of a low-level nonspecific esterase activity in the lysozyme preparation. Only trace proteolytic activity was detected in lysozyme preparations using denatured hemoglobin, casein, azocasein, albumin, or azoalbumin as substrates. Compared with lysozyme, a nonspecific protease preparation from *S. griseus* exhibited approximately 300-fold greater activity on the same substrates (data not shown). No hydrolysis was shown with synthetic esters used in the assay of trypsin, chymotrypsin, or carboxypeptidase A or B (data not shown). The nonspecific esterase activity was not inhibited by 50 mM *N*-acetylglucosamine, a competitive inhibitor of lysozyme lytic activity (21). In addition, the esterase activity/lysozyme lytic activity ratios were nearly identical for several different commercial batches of lysozyme at different states of purity (data not shown). $NaDodSO_4$ electrophoresis of purified lysozyme on gradient gels was monophoretic. The esterase and lytic activities were not separable by electrophoresis, gel filtration, or adsorption chromatography under a variety of conditions of buffer, pH, and ionic strength (data not shown).

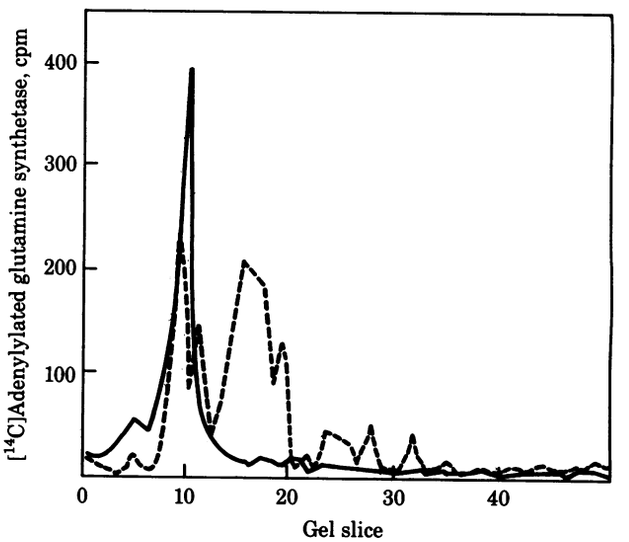

FIG. 3. Native pore gradient electrophoresis of 31 μg of [^{14}C]-adenylylated glutamine synthetase after incubation with 5 μg of lysozyme. ——, Zero time sample; – – –, sample incubated for 180 min at 37°C.

Carboxymethylation of Lysozyme. Piszkiewicz and Bruice (4) have reported that lysozyme possesses an inherent esterase activity that is at a catalytic site distinct from the lysozyme lytic site. They demonstrated that the lysozyme esterase site involved threonine-89 and histidine-15, the only histidine residue in lysozyme. To determine whether the esterase site might be responsible for the proteolysis observed with lysozyme preparations and lysozyme-prepared extracts, the histidine-15 residue was specifically modified by carboxymethylation, as described by Piszkiewicz and Bruice (4). A product was obtained that behaved chromatographically and enzymatically like the modified lysozyme described by those investigators. The modified product (CM-lysozyme) possessed no detectable esterase activity but retained 40% lysozyme lytic activity (Table 1). Also, CM-lysozyme is adsorbed on a Bio-Rex 70 column previously equilibrated with 50 mM phosphate buffer (pH 7.18). When a linear phosphate gradient to 200 mM was applied, CM-lysozyme eluted at approximately 150 mM phosphate, several fractions ahead of the unmodified lysozyme. Comparison of the amino acid compositions of CM-lysozyme and the unmodified lysozyme confirmed that carboxymethylation had led to loss of histidine and the formation of 3-carboxymethylhistidine and a small amount of 1,3-dicarboxymethylhistidine but no 1-carboxymethylhistidine. There was no change in methionine content. Only a slight change in the cysteine content was detected; however, cysteine is not well quantitated by the procedure used here and, in addition, there is partial positional overlap of cys-

Table 1. Effect of carboxymethylation on lysozyme lytic activity and lysozyme esterase activity

	% lytic activity	% esterase activity
Lysozyme	100	100
CM-lysozyme	40	0

Lytic activity was determined on *Micrococcus lysodeikticus* and esterase activity was determined on *p*-nitrophenylacetate.

teine with 3-carboxymethylhistidine on amino acid analysis. No other changes in amino acid content were observed and the amino acid composition agrees well with other published results (22).

When [^{14}C]adenylylated glutamine synthetase was incubated with the CM-lysozyme (Fig. 4*C*) or with CM-lysozyme-prepared extracts (data not shown), no proteolysis of the glutamine synthetase occurred. In both cases, the [^{14}C]adenylylated glutamine synthetase profile was identical to the profile of labeled glutamine synthetase alone (Fig. 4*A*). However, incubation of labeled glutamine synthetase with unmodified lysozyme (Fig. 4*B*) or with lysozyme-prepared extracts (data not shown) yielded multiple fragments. These results show that the proteolysis of glutamine synthetase observed in lysozyme-prepared extracts of *E. coli* is caused by lysozyme. The capacity of lysozyme to degrade glutamine synthetase is lost when the lysozyme esterase activity is inactivated by carboxymethylation of the histidine residue involved in the esterase site.

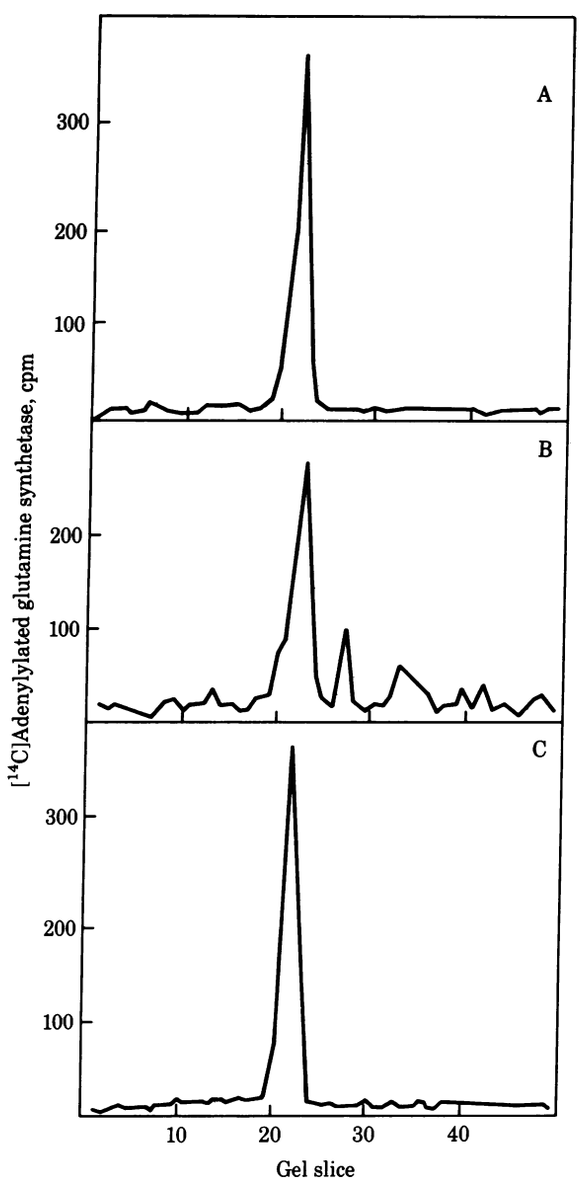

FIG. 4. NaDodSO$_4$ pore gradient electrophoresis of glutamine synthetase/lysozyme incubation mixtures. Thirty-one-microgram samples of [^{14}C]adenylylated glutamine synthetase were incubated alone (*A*), with 5 μg of lysozyme (*B*), or with 5 μg of CM-lysozyme (*C*) at 37°C for 180 min.

DISCUSSION

Incubation of cells with lysozyme in the presence of either EDTA (2) or high salt (3) is a convenient and perhaps the most gentle procedure for the preparation of bacterial extracts. For these reasons, this procedure has been widely used and, in particular, has been utilized in the search for proteolytic enzymes involved in protein turnover (1). The demonstration that lysozyme preparations possess an intrinsic proteolytic activity emphasizes the need for caution in the interpretation of results obtained with lysozyme prepared extracts, especially when such extracts are used for proteolytic enzyme studies.

Our results are reminiscent of the earlier work of Piszkiewicz and Bruice (4), who showed that lysozyme possesses an esterase site that is inactivated by carboxymethylation of the histidine-15 residue. The demonstration here that carboxymethylation leads to simultaneous loss of esterase and protease activity but only partial loss of lysozyme lytic activity suggests that the protease and esterase activities are properties of a common site that is topographically distinct from the lysozyme lytic site. This is also indicated by the fact that 50 mM *N*-acetylglucosamine, a competitive inhibitor of the lytic activity, has no effect on the esterase or protease activities.

There is disagreement concerning the position of the carboxymethyl modification of histidine and the role that histidine has in lysozyme lytic activity (4, 23, 24). Our results agree with those of Piszkiewicz and Bruice (4). In contrast, to the finding of Goux and Allerhand (24), we observed that carboxymethylation leads to partial loss of lysozyme lytic activity and suggest that this might be due to limited modification of a cysteine residue, which may occur under conditions used to modify the histidine residue. We observed no change in methionine or any other amino acid.

The fact that glutamine synthetase is degraded in extracts prepared by lysozyme treatment but not in extracts prepared by sonic oscillation and the further demonstration that purified lysozyme by itself will catalyze the degradation of glutamine synthetase suggests that most of the glutamine synthetase degradation that occurs in lysozyme-prepared extracts is due to a direct action of lysozyme. However, in subsequent experiments, we have detected the presence of a glutamine synthetase degradation system in which glutamine synthetase is first inactivated by a catalase-sensitive mixed-function oxidation reaction and thereby rendered susceptible to proteolysis by endogenous proteases (25).

It is noteworthy that lysozyme exhibits only trace proteolytic activity on a variety of protein substrates but causes considerable proteolysis of glutamine synthetase in relatively short incubation periods. Therefore, the interaction of lysozyme with glutamine synthetase might be somewhat specific. Indeed, it has been observed that, at low salt concentration, lysozyme precipitates glutamine synthetase, and that the insoluble complex can be dissociated by very high salt concentrations (unpublished data). Because lysozyme is a very basic protein (pI = 11.6), its coprecipitation with glutamine synthetase (pI = 4.7) might be the result of charge–charge interactions. The high sensitivity of glutamine synthetase to proteolysis by lysozyme may therefore be due to the high affinity of one for the other.

The proteolytic degradation of β-galactosidase in *E. coli* has been used as a model for studies of protein turnover. It is therefore noteworthy that β-galactosidase is also readily proteolyzed by lysozyme (data not shown). Moreover, the proteolysis of β-galactosidase and glutamine synthetase are both stimulated by 1 mM ATP, but the mechanism of the ATP effect is unclear. The ATP stimulation of β-galactosidase in lysozyme-prepared *E. coli* cell-free extracts was previously reported (2). An ATP-depen-

dent protease La [also called lon, cap R, deg (26)] has been purified from cell-free extracts prepared by French pressure cell disruption; this protease catalyzes the degradation of methylated casein or globin but has little or no activity on native protein substrates (26). Whether "La," lysozyme, or another protease is responsible for the degradation of β-galactosidase in lysozyme-prepared cell-free extracts remains to be ascertained.

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