Intracellular proteolytic degradation of glutamine synthetase occurs in two distinct steps in Escherichia coli (Levine, R. L., Oliver, C. N., Fulks, R. M., and Stadtman, E. R. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 2120–2124). In the first step, a mixed function oxidation modifies the glutamine synthetase. The modified enzyme, which is catalytically inactive, becomes susceptible to proteolytic attack. In the second step, a protease specific for the modified enzyme catalyzes the actual proteolytic degradation. The oxidatively modified glutamine synthetase was studied to determine the chemical differences between it and the native enzyme. Only a single alteration was found; one of sixteen histidine residues/subunit was altered by the oxidative modification. The modification introduced a carbonyl group into the protein, permitting isolation of a stable modification. The modification introduced a carbonyl group into the protein, permitting isolation of a stable modification.

The biochemical pathways for intracellular protein turnover remain essentially un mapped. We recently demonstrated that in bacteria, the proteolytic degradation of glutamine synthetase (L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2) involves two steps (2). First, the enzyme undergoes an oxidative modification which abolishes enzymic activity. Then a protease, specific for the modified enzyme, catalyzes degradation of the protein. The first step requires oxygen and a metal and is mediated by several enzymic and nonenzymic systems (2, 3). The accompanying paper reports detailed studies of one system, consisting simply of oxygen, ascorbate, and trace metal (4). Those studies demonstrated the potential for metabolic regulation of the oxidative modification of glutamine synthetase. Specifically, substrate availability and the extent of adenylylation of the enzyme dramatically affect the susceptibility of glutamine synthetase to oxidative modification. Thus, the first step in intracellular proteolysis becomes subject to regulatory control.

Although the oxidative modification abolishes enzymic activity and renders the protein susceptible to degradation, the alteration does not cause major changes in structure (2). The protein retains its dodecameric structure, and the constituent subunits retain their native molecular weight of 50,000. I report here that oxidative modification alters only a single histidine residue in the glutamine synthetase subunit. This specific alteration may "mark" the enzyme for intracellular proteolytic degradation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Pierce Chemical Co. supplied constant boiling HCl and amino acid calibration standard "H." HEPES was from Aldrich and replaced the 2-methyl imidazole buffer used earlier (2). Eastman Kodak Co. produced the 2,4-dinitrophenylhydrazine. Most amino acids and 5,5'-dithio-bis-(2-nitrobenzoic acid) were products of Sigma. The γ hydroxyglutamate was a gift from Dr. A. Meister (Department of Biochemistry, Cornell University Medical College, New York, NY). The α-aminol, α-d-hydroxylate was a gift from Dr. B. Seto (National Institutes of Health) (5).

**Methods**—Purification of native glutamine synthetase and preparation of the oxidatively modified synthetase were described previously (2). The average state of adenylylation of enzyme used here was low (<1). For preparation of larger amounts of concentrated inactivated protein (>10 mg/ml), 100–250 μM FeCl3 was added to increase the rate of inactivation. For amino acid analyses, proteins were hydrolyzed at 155 °C (6) with a benchtop system (7). Hydrolysis times were varied from 20 to 60 min, corresponding to the usual 24 to 72 h at 110 °C. To improve accuracy of analysis, samples were diluted after hydrolysis with 9 volumes of 600 mM NaOH, thus avoiding lyophilization (6). For the experiment shown in Fig. 2, the hydrolysis sample contained ascorbate, but control experiments showed that its presence did not affect the amino acid analysis.

The amino acid analyses were run on a Dionex model D-300 analyzer (Sunnyvale, CA) with fluorometric detection of α-phthalaldehyde derivatives. The manufacturer supplied a sample loop with a 20-μl nominal volume. Calibration with titrated water gave an actual volume of 30 μl. Typical samples were about 25 μM in histidine, so that the sample loop contained about 1 nmol of the amino acid. Because accuracy of analysis was critical in these studies, the basic amino acids were determined in a separate run from the acidic/neutral (6). Buffer systems were chosen so that histidine would be included in both the acidic/basic and natural runs. Isonitremic buffers and the α-phthalaldehyde were made as described by Hare (6), with the addition of sodium phosphate, pH 7.35 (8). The basic chromatogram was developed isocratically with 367 mM sodium citrate, pH 5.26. The integrator was a Shimadzu Chromatopac C-R1A (Kyoto, Japan) which was calibrated with 12.5 and 25.0 μM standards. This 2-point calibration is essential for accuracy, as it allows for nonzero intercepts. After calibration in this fashion, the integrated areas were strictly linear with concentrations from 2.5 to over 50 μM for each amino acid and ammonia. The correlation coefficient for the linear regression was >0.99 in every case. Fig. 1 shows the curve for histidine, with 95% confidence limits (9).

The glutamine synthetase subunit contains 16 histidine residues (10). Using these analytical techniques, a loss of one of 16 histidines between proteins is readily quantitated. This was confirmed by adding

* A preliminary report of these studies appeared in abstract form (1). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Oxidation of Histidine in Glutamine Synthetase

Fig. 1. Linear response of the amino acid analyzer to the concentration of histidine. Serial dilutions of the amino acid standard were analyzed as described under "Experimental Procedures." The fit regression equation was $\text{mV-sec} = -1.658 + 9.1029 \times [\text{histidine}]$ with $r > 0.999$. The lines are the 95% confidence limits (9).

an aliquot either of the Pierce amino acid standard solution or of buffer to a hydrolysate of glutamine synthetase. The amount added was chosen to give a calculated difference of one part in 16 for the histidine concentrations between the "spiked" and the buffer samples. The calculated difference was 1.25 mM. Analogous analyses gave 15.65 mM histidine in the spiked sample and 17.38 mM in the buffer sample, a difference of 1.72 mM. Taking the spiked sample as 100.0%, the buffer sample was 95.2%. Thus, a difference of one of 16 histidines was readily demonstrated. A similar analysis confirmed that loss of one of 16 tyrosine or one of 24 phenylalanine residues could be detected.

The concentrations of individual amino acids in a protein hydrolysate will not be equal, as they are in the calibrating standards. Depending on the method of integration, this could introduce small errors in the analysis of the protein hydrolysate, especially for incompletely resolved amino acids such as threonine and serine. One should be able to avoid this potential problem in comparing the amino acid composition of the native enzyme with the ascorbate-treated enzyme.

Aromatic Amino Acids—Oxidative modification of glutamine synthetase induces a UV difference spectrum which is qualitatively and quantitatively consistent with hydroxylation of a phenolic moiety (2). However, acid hydrolysis and amino acid analysis revealed no difference in the phenylalanine and tyrosine contents of native and oxidatively modified proteins (Table I). No dopa or similar catechol was found. Also, neither of the two tryptophan residues was altered, as determined by alkaline hydrolysis (Table I) and by the fluorescence emission spectra of the proteins. As noted under "Experimental Procedures," amino acid analysis should have detected loss of a single phenylalanine or tyrosine residue (24/subunit) and 16/ subunit, respectively (10). The hydrolysis results were confirmed using a sensitive nondestructive technique, multicomponent analysis of the UV second derivative spectrum (13). Table 1 also shows those results, confirming that oxidative inactivation does not involve alteration of the aromatic amino acids.

Cysteine and Methionine—The sulfur-containing amino acids are likely candidates for oxidative modification (14). Titration of the protein with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) showed that both native and modified proteins contained 4 cysteine residues (Table II). (Glutamine synthetase does not contain dsulfide bonds (10).) Amino acid analysis of acid hydrolysates revealed no difference in methionine content. However, methionine sulfoxide can revert to

**TABLE I**

| Amino acid | Native | Modified | Modified/native |
|------------|--------|----------|-----------------|
| Phenylalanine | 24.0 | 23.2 | 1.00 |
| Tyrosine | 17.4 | 17.6 | 1.01 |
| L-Tryptophan | 1.95 | 1.92 | 1.02 |
| Phenylalanine | 21.9 | 21.8 | 1.00 |
| Tyrosine | 16.3 | 16.2 | 0.99 |
| L-Tryptophan | 2.07 | 2.05 | 0.99 |

*By amino acid analysis of an acid hydrolysate.

**TABLE II**

| Amino acid | Native | Modified | Modified/native |
|------------|--------|----------|-----------------|
| Cysteine | 3.83 | 3.84 | 1.00 |
| L-Methionine | 15.0 | 14.6 | 0.97 |
| L-Methionine sulfoxide | <0.6 | <0.6 | 1.00 |

* As noted under "Experimental Procedures," cysteine determination was performed in 6 M guanidine. Without denaturation, neither native glutamine synthetase (10) nor the modified protein react with 5,5'-dithiobis-(2-nitrobenzoic acid).

Methionine shows somewhat more variability than the other amino acids, with a slight decrease in yield as hydrolysis time is increased, presumably due to oxidation during hydrolysis. However, no consistent differences between the native and modified proteins were observed. The results here are from a 30-min hydrolysate.

The apparent traces of methionine sulfoxide could result from less than 100% efficiency of attack of cyanogen bromide on methionine (13).
methionine during acid hydrolysis (15). Methionine sulfoxide formation may be an important reversible covalent modification of proteins (16). The cyanogen bromide technique was used to check for possible methionine sulfoxide formation, with acid hydrolysis as described above (17). None was present (Table II).

Loss of One Histidine Residue—Having ruled out changes in the aromatic and sulfur-containing residues, the full amino acid analyses of the native and oxidatively modified proteins were compared (Table III). Analyses of five separate pairs of proteins always revealed the same change; oxidatively modified glutamine synthetase lost one of 16 histidine residues.

While alteration of a histidine residue is the only detected change in the modified enzyme, this need not be the alteration which causes loss of catalytic activity. There could be another undetected alteration which abolishes enzymic activity. To study this possibility, glutamine synthetase was exposed to the ascorbate system for varying times. This generated a series of proteins with graded loss of catalytic activity. Amino acid analyses demonstrated that loss of catalytic activity did parallel loss of the histidine residue (Fig. 2).

Generation of a New Amino Acid—Oxidation of histidine

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\text{TABLE III}
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| Amino Acid | Modified/native | Amino Acid | Modified/native |
|------------|-----------------|------------|-----------------|
| Aspartate  | 1.005           | Tyrosine   | 1.012           |
| Threonine  | 0.996           | Phenylalanine | 0.991         |
| Serine     | 0.996           | Lysine     | 1.013           |
| Glutamate  | 1.012           | Histidine  | 0.902           |
| Glycine    | 0.989           | Arginine   | 0.984           |
| Alanine    | 1.003           | Mean:      | 1.000           |
| Valine     | 1.017           | S.D.:      | 0.015           |
| Methionine | 0.983           |            |                 |
| Isoleucine | 1.016           |            |                 |
| Leucine    | 1.009           |            |                 |
| Tyrosine   | 1.021           |            |                 |
| Phenylalanine | 1.001       |            |                 |
| Histidine* | 0.943           |            |                 |
| Mean:      | 1.000           | S.D.:      | 0.020           |

* Only the content of histidine differs significantly between the two proteins. To normalize, the initial ratios were divided by the average of all the ratios. For the acidic/neutral run this was 0.9568; for the basics it was 1.189. This table shows the results from 45-min hydrolysates. The same conclusion resulted from 15-, 30-, and 60-min hydrolysates.

Fig. 2. Loss of one histidine residue parallels the loss of glutamine synthetase activity. O, the enzymatic activity; x, the histidine residue. Note that 0% remaining histidine means the loss of one histidine residue out of a total of 16 in the glutamine synthetase subunit.

May generate a bewildering array of products, including aspartate and glycine (18). The latter products would not be detected with amino acid analyses because of the large background content. However, a new α-phthalaldehyde-reactive product was consistently noted, eluting between serine and glutamate (Fig. 3). Using the partially inactivated preparations of glutamine synthetase, it was found that the generation of this unknown product also parallels loss of catalytic activity (Fig. 4). While rather stable to acid hydrolysis, maximal yield of this compound obtained after about 30 min of acid hydrolysis at 155 °C. At 60 min, a 10–20% decrease in yield occurred. It has an elution time close to, but distinguishable from, homoserine and α-amino, δ-hydroxyvaleric acid (Table IV). These chemical and chromatographic characteristics suggest that the unknown product may be a hydroxylated amino acid.

In the detection system used here, the fluorescence yield of the acidic and neutral amino acids are virtually identical (Ref. 19 and confirmed by our own data). Assuming that the unknown product has the same fluorescence yield, one can estimate that it represents about one-fifth to one-fourth of a residue/subunit glutamine synthetase. This is consistent with the multiple products expected upon acid hydrolysis of the oxidatively modified histidine.

Does the Oxidative Modification Occur in Vivo?—Photooxidation of N-benzoylhistidine can introduce a carbonyl group...

Fig. 3. Amino acid analyzer tracing for the hydrolysate of ascorbate-inactivated glutamine synthetase. To optimize separation of the unknown amino compound, the chromatogram was developed isocratically with 67 mM sodium citrate, pH 3.0, at 31 °C. The sensitivity of the recorder was increased 64-fold between the markers (v).

Fig. 4. Appearance of the unknown product parallels loss of glutamine synthetase activity. O, the enzymatic activity; x, the unknown product, quantitated with the chromatographic analysis shown in Fig. 3. The highest value (at 121 min) was set to 100%.
Oxidation of Histidine in Glutamine Synthetase

The ascorbate system was studied because it appears to be a good model for the enzymic systems which exist (2, 3) and because the modified protein could be obtained in large amounts in pure form. The other in vitro systems presumably lead to the same modification as the ascorbate system.

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Zymase as a reference (Fig. 5). Thus, the modification introduced by the ascorbate system also occurs in enzymes isolated with our usual purification scheme. We doubt that the oxidative modification occurs during isolation, because all steps of the purification procedure include a manganese concentration which should block oxidation (2). Thus, the oxidative modification probably occurred in vivo or during storage of the cells after culture.

DISCUSSION

Oxidative modification of glutamine synthetase by the ascorbate system causes loss of catalytic activity and "marks" the protein for subsequent proteolytic degradation (2, 37). As shown here, the modification leads to a loss of one of 16 histidine residues in the glutamine synthetase subunit. Radiation or photosensitized oxidation of histidine residues rarely show such specificity. The specificity in this nonenzymic system implies that the modified histidine lies within a distinctive site in the enzyme, rendering the residue particularly susceptible to oxidative modification. The modification of a histidine can explain why the ascorbate-mediated reaction displays a pH optimum close to neutrality, even though a neutral pH slows the oxidation of ascorbate (21). Histidine effectively protects glutamine synthetase against oxidative modification (4). This could be due to competition for the oxidizing species, or it might simply result from chelation of the metal which is required for oxidation of the protein (2).

While the modified histidine residue is clearly essential for catalytic activity, it need not lie at the active site. Glutamine synthetase must bind divalent cations to be catalytically active, and several binding sites are known (10). Oxidation of the histidine might destroy one of these sites, thereby preventing binding of the essential cation. Studies of the cation-binding properties of the modified protein should be informative.

The ascorbate system was studied because it appears to be a good model for the enzymic systems which exist (2, 3) and because the modified protein could be obtained in large amounts in pure form. The other in vitro systems presumably lead to the same modification as the ascorbate system. The reaction of naturally occurring glutamine synthetase preparations with 2,4-dinitrophenylhydrazine suggests that the same chemical modification may occur in vivo.

It seemed possible that certain other enzymes might prove susceptible to oxidative modification. Noting that the modification requires a divalent cation and that a histidine is altered, certain kinases and dehydrogenases appeared candidates for oxidative modification. (Many of these enzymes have a histidine at their active site and require divalent cations for catalytic activity (22, 23).) Enzymes such as pyruvate kinase, creatine kinase, lactate dehydrogenase, and alcohol dehydrogenase did prove susceptible to oxidative inactivation (36). In the case of NADH oxidase-mediated inactivation of yeast 3-phosphoglycerate kinase, we found that the inactivated enzyme had lost one of nine histidine residues (36). Inactivation of superoxide dismutase by hydrogen peroxide causes loss of one histidine residue (24). Recently, Lerch noted that catechol specifically modifies the histidine at position 306 in tyrosinase (25). Thus, the oxidation of histidine emerges as a common and potentially important covalent modification of proteins.

The biological roles for such a covalent modification remain speculative. We did show that oxidatively modified glutamine synthetase is subject to proteolytic degradation by extracts of Escherichia coli while the native enzyme is spared (2, 37). The oxidation of the imidazole ring of a histidine could render the

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**Table IV**

| Amino acid                  | Elution time (min) |
|-----------------------------|--------------------|
| β-Hydroxyglutamic           | 13.04              |
| Aspartic                    | 13.22              |
| γ-Hydroxyglutamic           | 13.40              |
| Thrreonine                  | 14.80              |
| Serine                      | 15.75              |
| Homoserine                  | 18.20              |
| Unknown                     | 18.83              |
| α-Amino, β-hydroxyvaleric   | 19.08              |
| Glutamic                    | 21.23              |
| Glycine                     | 26.15              |
| Alanine                     | 28.35              |

**Fig. 5.** 2,4-Dinitrophenylhydrazone formation is proportional to loss of glutamine synthetase activity. ×, ascorbate-treated enzymes; O, purified preparations not exposed to ascorbate. As explained under "Experimental Procedures," hydrazone formation is expressed as the difference in molar absorptivity at 387 and 400 nm. The molar absorptivity is referred to the concentration of glutamine synthetase subunits. The regression equation is hydrazone formation = 693 - 4.90 x (specific activity), with r = -0.986. The x intercept is 141.5 units/mg, representing the specific activity of unmodified enzyme. The most active preparations of glutamine synthetase isolated in this laboratory over 20 years have had specific activities of about 140 units/mg.

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* R. L. Levine and S. Shaltiel, unpublished data.
adjacent peptide bond more labile to cleavage, as shown by Shaltiel and Patchornik with small peptides (26). A protease specific for the modified synthetase has been partially purified (2, 37, 38). So, oxidative modification of glutamine synthetase may indeed “mark” it for subsequent proteolytic degradation. The accompanying paper documents the potential for metabolic regulation of the oxidative step, strengthening the possibility that the reaction is of physiologic significance (4, 36).

Cytochrome P-450-dependent systems mediate the oxidative modification of glutamine synthetase in vitro, and we have suggested that cytochrome P-450-dependent oxidases may be involved in intracellular proteolysis (2). Interestingly, Paine and Francis found that certain oxidation products of histidine induce a cytochrome P-450-dependent oxygenase (27).

Glutamine synthetase occupies a critical position in the metabolic pathways of bacteria (10). Mammalian host defense systems could take advantage of this potential Achilles’ heel. Upon stimulation by bacterial invasion, mammalian white cells undergo an “oxidative burst” with production of diffusible species of activated oxygen and hydrogen peroxide (28–30). If these products reach the bacterial glutamine synthetase, they may cause oxidative inactivation with potentially lethal disruption of bacterial metabolism. Preliminary experiments demonstrate that incubation of intact cells of E. coli with a cultured neutrophil line causes loss of bacterial glutamine synthetase activity (36). Oxidative inactivation of enzymes may also serve to protect higher organisms from autolysis by lysosomal enzymes released from their own activated neutrophils. Evidence for this protective function comes from studies by Voetman et al., who showed that phagocytosing neutrophils do inactivate their own lysosomal enzymes in an oxygen-dependent reaction (31).

Decreases in the specific activity of many enzymes occur during aging (32). The mechanisms which cause this change are unknown, but oxidative modifications could be responsible (32, 33). Since oxidized proteins may be more rapidly degraded, evaluation of their significance to development and aging might be difficult. However, proteins in the lens of the eye are stable. They do not undergo proteolysis, and detection of oxidative changes in lens proteins should be facilitated. Indeed, aged and cataractous lenses do have an increased content of oxidized proteins (34). Finally, oxidative changes in proteins may be a cause of oxygen toxicity in patients who receive oxygen therapy. The toxic side effects of oxygen therapy are of particular concern in prematurely born infants who require oxygen supplementation. In these babies, oxygen therapy may cause blindness (retrolental fibroplasia) or a potentially fatal pulmonary disorder (bronchopulmonary dysplasia) (35).

Further studies on the oxidative modification should be facilitated by isolation of a peptide containing the modified histidine (39) and by the use of immunologic or chemical probes such as 2,4-dinitrophenylhydrazine or the unknown amino acid generated upon inactivation. Reversal of the oxidative modification has not yet been demonstrated, but such a reversal would increase the potential metabolic flexibility of this covalent modification.

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