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Peroxynitrite-mediated nitration of tyrosine residues in *Escherichia coli* glutamine synthetase mimics adenylylation: Relevance to signal transduction.

(nitrotyrosine)

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ABSTRACT **Treatment of *Escherichia coli* glutamine synthetase (GS) with peroxynitrite leads to nitration of some tyrosine residues and conversion of some methionine residues to methionine sulfoxide (MSOX) residues. Nitration, but not MSOX formation, is stimulated by Fe-EDTA. In the absence of Fe-EDTA, nitration of only one tyrosine residue per subunit of unadenylylated GS leads to changes in divalent cation requirement, pH–activity profile, affinity for ADP, and susceptibility to feedback inhibition by end products (tryptophan, AMP, CTP), whereas nitration of one tyrosine residue per subunit in the adenylylated GS leads to complete loss of catalytic activity. In the presence of Fe-EDTA, nitration is a more random process: nitration of five to six tyrosine residues per subunit is needed to convert unadenylylated GS to the adenylylated configuration. These results and the fact that nitration of tyrosine residues is an irreversible process serve notice that the regulatory function of proteins that undergo phosphorylation or adenylylation in signal transduction cascades might be seriously compromised by peroxynitrite-promoted nitration.**

Regulation of glutamine synthetase (GS) activity in *Escherichia coli* is mediated by the cyclic attachment and detachment of the AMP moiety of ATP to the hydroxyl group of a unique tyrosine residue in each of the 12 identical subunits of the enzyme. The adenylylation and deadenylylation of GS are catalyzed at separate sites on a single adenylyl transferase, whose activities are under strict allosteric control by metabolites, and by interactions with the uridylylated and unmodified forms of the P_{II} regulatory protein (1, 2). Among other properties, adenylylation of GS leads to a shift in the pH–activity profile, alterations in the divalent cation specificity, a change in affinity of the enzyme for its substrates (especially ADP), and changes in susceptibility of the enzyme to feedback inhibition by multiple end products of glutamine metabolism. In an earlier study, it was demonstrated that treatment of GS with tetranitromethane led to nitration of about two tyrosine residues per subunit and to conversion of the unadenylylated enzyme to a form similar to that produced by adenylylation (3).

In the meantime, it has been shown that peroxynitrite (PN) can be formed endogenously by the interaction of nitric oxide with superoxide anion and that PN can react with tyrosine residues of proteins to form nitrotyrosine derivatives (4, 5). We show here that exposure of unadenylylated GS to PN leads to nitration of tyrosine residues and to conversion of the enzyme to a form that is similar to the adenylylated enzyme, whereas nitration of the adenylylated enzyme leads to complete loss of catalytic activity. A parallel study in this laboratory (17) demonstrated that nitration of the tyrosine residue of a synthetic peptide patterned after the phosphorylation site of $p34^{cdc2}$, a substrate for lck kinase ($p56^{lck}$), prevents phosphorylation of the peptide by this enzyme. These results and the consideration that nitration is an irreversible process underscore the fact that physiological conditions that favor the production of PN might seriously compromise one of the most important mechanisms of cellular control: signal transduction networks.

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MATERIALS AND METHODS

Materials. GS was from *Escherichia coli YMC 10 pgln6*, which overproduces the enzyme. The enzyme was purified by the zinc aggregation procedure as described (6).

PN. For some experiments, PN was prepared by reaction of H_2O_2 with $NaNO_2$ at 1°C in strong acid followed by the addition of excess base. The excess H_2O_2 was removed by treatment with MnO_2 as described (7). For most experiments PN was synthesized by the reaction of NaN_3 with ozone as described by Pryor *et al.* (8). The final stock solution, pH 10, contained 40 mM PN as indicated by its absorbance at 302 nm (ε_M = 1670).

The State of Adenylylation. The average number of adenylylated subunits per dodecamer of GS was determined as described (9) by measuring the γ-glutamyltransferase activity at pH 7.57 in the presence of 0.04 mM Mn(II) and also in the presence of 0.04 mM Mn(II) plus 60 mM Mg(II) or alternatively by measuring the transferase activity in the presence of 0.04 mM Mn(II) at both pH 7.57 and pH 9.0. These methods take advantage of the fact that adenylylated and unadenylylated subunits have identical activities in the presence of Mn(II) at pH 7.57, whereas only the unadenylylated enzyme is active at high concentrations of Mg(II) at pH 7.57 or in the presence of Mn(II) at pH 9.0.

The reactions of PN with GS were carried out as follows: 1.0-ml reaction mixtures containing 1 mg of GS, 50 mM potassium phosphate buffer (pH 6.0 to 7.5, as indicated), 0.1 M KCl, and PN at concentrations ranging from 0 to 5 mM, as indicated, were incubated at 37°C. Although the reaction was over almost instantaneously, for convenience of multiple analyses, the reaction mixtures were analyzed after 20 min as follows:

(i) GS activity. Ten microliters of the reaction mixture was added to 240 μl of H_2O, and 20 μl (0.8 ng of protein) of the diluted sample was taken for assay of GS activity in three

Abbreviations: GS, glutamine synthetase; PN, peroxynitrite; MSOX, methionine sulfoxide; $\bar{n}$, number of adenylylated subunits per dodecamer of GS; $\bar{n}$-app, apparent number of adenylylated subunits per dodecamer of GS.

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different assay mixtures—namely, in mixtures containing either 0.04 mM Mn(II) at pH 7.57, or 0.04 mM Mn(II) plus 60.0 mM Mg(II) at pH 7.57, or 0.04 mM Mn(II) at pH 9.0, and the state of adenylylation ($\bar{n}$) was calculated as described (9), where $\bar{n}$ is the number of adenylylated subunits per dodecamer. In the present study, we show that nitration of tyrosine residues provokes changes similar to those associated with adenylylation. Accordingly, instead of $\bar{n}$, we refer to the changes as apparent changes in the state of adenylylation ($\bar{n}$-app).

(ii) Nitrotyrosine. The reaction mixtures were subjected to centrifugation in a Centricon 10 to separate protein from small molecules. The protein fraction was washed three times with H_2O and then once with standard buffer containing 10 mM potassium phosphate (pH 7.4), 10 mM Mg(II), and 100 mM KCl. Fifty microliters of the concentrated protein (3–6 mg of protein per ml) was added to a quartz cuvette and diluted to 0.5 ml with H_2O, and the spectrum was recorded from 200 to 600 nm, before and after addition of 0.01 ml of 1.0 M NaOH, using a Cary 4 UV-VS spectrophotometer. The concentration of GS protein was calculated from the absorbance at 278 nm before NaOH addition (A_{278} = 0.733 per mg of GS protein per ml). The amount of nitrotyrosine was calculated from the absorbance at 428 nm after NaOH addition (ε_M = 4100).

(iii) Methionine sulfoxide (MSOX). Ten micrograms of the Centricon-treated protein was incubated overnight with 100 μl of cyanogen bromide/formic acid solution (10 μl of 1.0 M cyanogen bromide plus 90 μl of 70% formic acid) at room temperature, in the dark, in a ventilation hood. The sample was then vacuum dried and subjected to acid hydrolysis in the presence of 1 mM dithiothreitol, and then the amino acid composition of the hydrolysate was determined as described (10–12). Under these conditions, authentic methionine residues are recovered as homoserine or homoserine lactone, and MSOX residues are recovered as methionine.

(iv) Multicatalytic protease. The multicatalytic protease was purified from rat liver acetone powder (Sigma) and assayed as described (18). The free amino groups on small peptides generated upon GS proteolysis by the multicatalytic protease were quantified using fluorescamine (Sigma) after protein precipitation with 10% trichloroacetic acid.

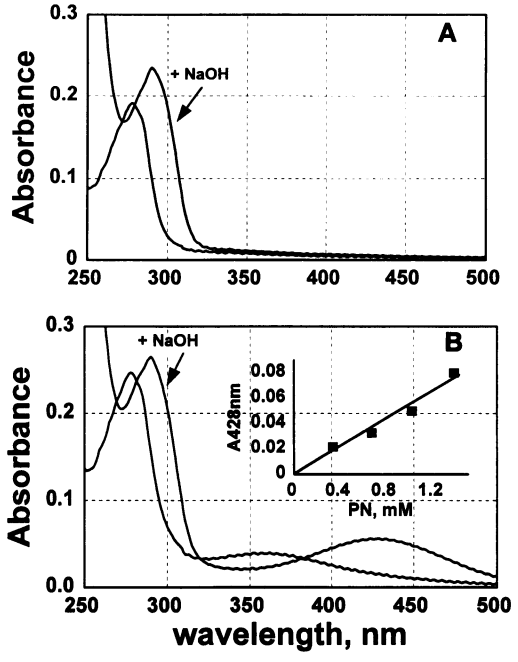

FIG. 1. Effect of nitration on the spectrum of GS. The reaction mixture of GS (0.5 mg/ml) with PN was prepared as described (7) in *Materials and Methods*. After incubation without PN (*A*) and with PN (*B*), the small molecules were separated from the protein by gel filtration through a P-10 column. The spectrum of the protein (0.15 mg/ml) was measured both before and after addition of NaOH. (*Inset*) Absorbance at 428 nm as a function of PN concentration.

RESULTS

Reaction of PN with Tyrosine, *o*-Phosphotyrosine, and GS. After reaction with PN, the spectrum of tyrosine at pH 12 exhibits a new absorption peak at 428 nm that is characteristic of *o*-nitrotyrosine, whereas the spectrum of *o*-phosphotyrosine is unaffected by the PN treatment (data not shown). This indicates that tyrosine, but not phosphotyrosine, can be nitrated by PN and is consistent with the fact that electrophilic substitution at the ortho position of esterified tyrosine derivatives is unfavorable. After treatment with PN, the spectrum of GS also exhibits an absorption peak at 428 nm (pH 12), showing that tyrosine residues have been nitrated (Fig. 1). Moreover, the increase in absorbance at 428 nm is a nearly linear function of the PN concentration over the range of 0.34 to 1.4 mM (Fig. 1 *Inset*).

Changes in Divalent Cation Specificity and Loss of Mn(II)-Dependent Activity at pH 9.0 Is Associated with Nitration of Tyrosine Residues. *(i) Loss of Mg(II)-dependent transferase activity.* Because the unadenylylated form of GS can utilize either Mg(II) or Mn(II) for transferase activity, whereas the adenylylated enzyme has an absolute requirement for Mn(II), and also because adenylylated and unadenylylated subunits act independently, the ratio of activity measured in the presence of Mg(II) to that measured in the presence of Mn(II) decreases linearly as the number of adenylylated subunits per dodecamer increases from 0 to 12 (9). A similar change in the Mg(II)/Mn(II) activity ratio occurs when GS is exposed to increasing concentrations of PN (Fig. 2). The fact that the activity ratio in the absence of PN treatment (zero nitrotyrosine residues) is about 0.8 reflects the fact that the GS preparation used in this study contained on the average about 2.0 adenylylated subunits per dodecamer.

It is evident from the data in Fig. 2 that, when GS is treated with PN in the presence of 0.1 M KCl, the relationship between tyrosine nitration and the Mg(II)-supported activity is not seriously affected by variations in pH over the range of 5.5 to 6.5 and is essentially independent of whether low concentrations (20–40 μM) of Fe(III)-EDTA or Mn(II) are present during the PN treatment. In all cases, extrapolation of the nitrotyrosine vs. the divalent cation-dependent activity ratio

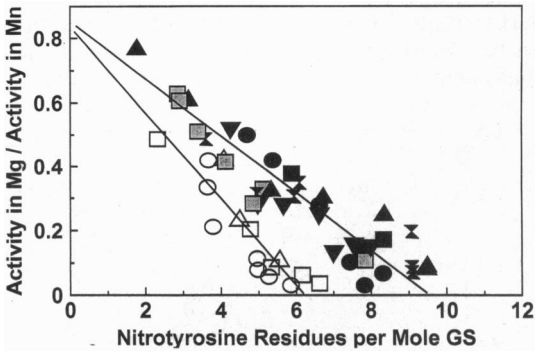

FIG. 2. Relationship between the Mg(II)/Mn(II) activity ratio and the number of nitrotyrosine residues formed per mole of GS dodecamer. After incubation of GS (1.0 mg/ml) with various PN concentrations (0–2 mM), the nitrotyrosine content and GS activity at pH 7.57 in the presence of 0.04 mM Mn(II) and also in 0.04 mM Mn(II) plus 60 mM Mg(II) were measured as described in *Materials and Methods*. The symbols refer to reaction mixtures containing 0.1 M KCl at pH 7.0 (○), pH 7.6 (△), pH 6.0 (▨), pH 6.3 (●), pH 5.5 (■), pH 6.0 in the presence of 20 μM Fe-EDTA (⧗), pH 6.0 in the presence of 20 μM Fe(III) and 40 μM EDTA (▲), pH 6.0 in the presence of 100 μM Mn(II) (▼), and in the absence of KCl at pH 6.0 (□).

shows that the ability of Mg(II) to support activity is lost when 10 tyrosine residues have been nitrated. A limiting value of 10, rather than 12, likely reflects the fact that the GS preparation used in this study contained about 2 adenylylated subunits per dodecamer. In contrast, when GS was treated with PN at neutral to alkaline pH values, or at pH 6.0 in the absence of KCl, nitration of only 6 tyrosine residues per mole abolished the Mg(II)-supported activity. The differential effects of pH and KCl on the relationship between nitration and loss of Mg(II)-supported activity could reflect differences in the modification of residues other than tyrosine (namely, methionine or cysteine residues) or differences in the conformational state of the protein (see *Discussion*). In any case, it is evident from the data in Fig. 2 that exposure of GS to PN leads to alterations in divalent cation specificity that are comparable to changes provoked by adenylylation of the enzyme.

(ii) Loss of Mn(II)-dependent activity at pH 9.0. Due to differences in the pH–activity profiles, the Mn(II)-supported transferase activity of unadenylylated subunits at pH 7.57 is almost identical to that obtained at pH 9.0, whereas adenylylated subunits are almost completely inactive at pH 9.0 in the presence of Mn(II). It follows that in the presence of Mn(II), the ratio of activity measured at pH 9.0 to that measured at pH 7.57 will decrease linearly from a value of 1.0 to nearly 0 as the number of adenylylated subunits per dodecamer is increased from 0 to 12 (9). As shown in Fig. 3, the pH 9.0/pH 7.57 activity ratio decreased from a value of 0.7 to 0 as the number of nitrotyrosine residues increased from 0 to about 10. Thus, as with adenylylation, nitration of about 1 tyrosine residue per subunit leads to almost complete loss of Mn(II) activity at pH 9.0.

Adenylylated GS Is Inactivated by PN. Treatment of adenylylated and unadenylylated forms of GS with increasing amounts of PN (0–4 mM) leads to parallel increases in the number of nitrotyrosine residues formed (Fig. 4). Nevertheless, the effect of nitration on the catalytic activity of these two forms of GS is very different. As shown in Fig. 4, the nitration of about seven tyrosine residues per dodecamer has little effect on the transferase activity of unadenylylated GS but leads to almost complete inactivation of the adenylylated enzyme. It is unlikely that the adenylylated tyrosine residues of the adenylylated GS are the site of nitration since PN is unable to nitrate *o*-phosphotyrosine. Whether the nitration of unadenylylated GS involves the nitration of tyrosine-397, the residue that is involved in the enzyme-catalyzed adenylylation of GS, remains to be established.

Effect of High Concentrations of Fe-EDTA. Beckman *et al.* (13) have reported that the rate of nitration of a model phenolic compound increases linearly as the concentration of

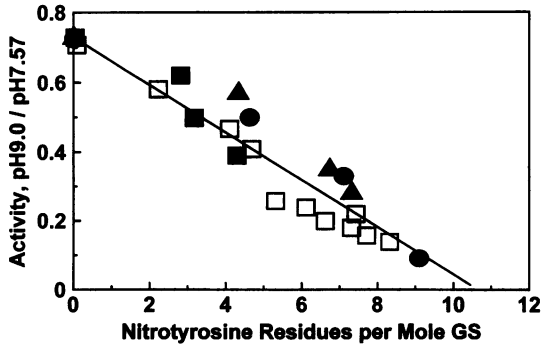

FIG. 3. Effect of nitration on the ratio of Mn(II)-dependent activity of GS at pH 7.57 to that at pH 9.0. Nitration of GS was in the presence of 0.1 M KCl as described in Fig. 2, but activity measurements were made in the presence of 0.04 mM Mn(II) at pH 7.57 and also at pH 9.0. The pH of reactions with PN were as follows: pH 5.5 (●), pH 6.0 (□), pH 6.3 (▲), pH 7.5 (■).

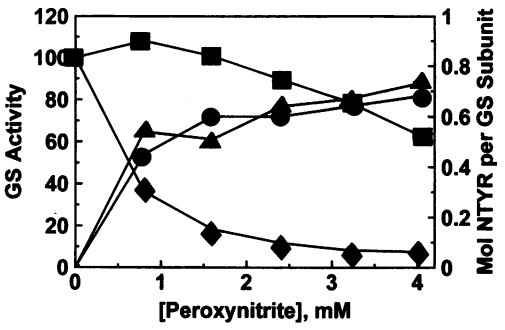

FIG. 4. Comparison of the effects of PN concentration on nitration and activity of adenylylated and unadenylylated forms of GS. Nitration of GS was carried out in the presence of 0.1 M KCl at pH 7.5 and the indicated concentration of PN. Symbols are as follows: ■, activity of unadenylylated GS; ◆, activity of adenylylated GS; ▲, nitrotyrosine (NTYR) of unadenylylated GS; ●, nitrotyrosine of adenylylated GS.

Fe-EDTA is increased over the range of 0 to 2 mM. At the low concentrations of Fe-EDTA used in the experiments summarized in Figs. 2 and 3, there was no detectable effect on either the extent of nitrotyrosine formation or the $\bar{n}$-app. However, as shown in Fig. 5*A*, at a given PN concentration, the nitration of tyrosine residues of GS increased almost linearly as the concentration of Fe-EDTA was increased from 0 to 0.5 mM. Moreover, as shown in Fig. 5*B*, at all Fe-EDTA levels the value of $\bar{n}$-app increased linearly as a function of the number of

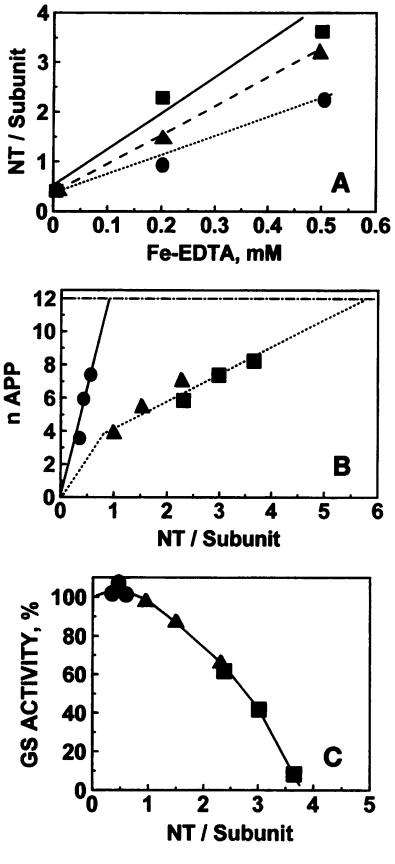

FIG. 5. Effect of high concentrations of Fe-EDTA on PN-mediated nitration and activity of GS. Reactions were carried out as described in Fig. 4 with the additional conditions indicated below. (*A*) Varying the concentration of Fe-EDTA with PN level maintained at 0.5 mM (●), 1.0 mM (▲), and 2.0 mM (■). (*B*) $\bar{n}$-app vs. the number of nitrotyrosine residues per subunit. Reaction mixtures contained either no (●), 0.2 mM (▲), or 0.5 mM (■) Fe-EDTA. (*C*) GS activity vs. the number of nitrotyrosine residues per subunit. Symbols are the same as in *B*.

Table 1. Effect of nitration on feedback inhibition of GS transferase activity

Form of GS	Relative activity		
	Trp	AMP	CTP
Unadenylylated	105	102	109
Adenylylated	71	75	6
Nitrated unadenylylated	74	40	2

Transferase activity was measured at pH 7.57 in the presence of 60 mM Mg(II) and 0.04 mM Mn(II), as described in *Materials and Methods*, except that the concentrations of glutamine and NH_2OH were 20 mM each and tryptophan (Trp), AMP, or CMP was 20 mM. Relative activity is expressed as the ratio of the activity observed in the presence of effectors to the activity observed in the absence of effectors.

nitrotyrosine residues formed per dodecamer of GS. Extrapolation of these linear segments to a value of $\bar{n}$-app = 12 (indicated by the dashed line in Fig. 5*B*) specifies the number of tyrosine residues that must be nitrated to convert GS from a form that mimics the adenylylated enzyme in which all 12 subunits contain 1 adenylylated tyrosine residue. In the absence of Fe-EDTA, nitration of 1.0 tyrosine residue per subunit converts GS to a form that is similar to that observed by adenylylation of all 12 subunits. In contrast, nitration of between 5 and 6 tyrosine residues per subunit is required when 0.2–0.5 mM Fe-EDTA is present. It is therefore evident that nitration in the absence of Fe-EDTA is a highly selective event; only 1 tyrosine residue per subunit is susceptible to nitration and nitration of that residue converts GS to a form that is similar to the adenylylated form. In the presence of Fe-EDTA, nitration is a more random process; nitration of 5–6 tyrosine residues per subunit is needed to convert GS to the adenylylated configuration. Furthermore, it is evident from the data in Fig. 5*C* that the nitration of more than 1 tyrosine residue per subunit leads to inactivation of GS; thus, the activity of GS declines to <10% of the initial value when 3.5–4 tyrosine residues per subunit are nitrated. Nevertheless, the $\bar{n}$-app of the residual activity increases as the number of nitrotyrosine residues increases (Fig. 5*B*).

It is noteworthy that Fe-EDTA had little, if any, effect on the conversion of methionine residues to MSOX residues (data not shown). Hence, the observed effect of Fe-EDTA on $\bar{n}$-app is most likely due to nitration of tyrosine residues.

Effect of Nitration on Feedback Inhibition of GS Transferase Activity. Several end products of glutamine metabolism (tryptophan, AMP, CTP) have little or no effect on the transferase activity of unadenylylated GS but partially inhibit the transferase activity of adenylylated GS (1, 2). This is illustrated by the data in Table 1, which also show that nitration of GS leads to a change in the feedback inhibition pattern that is similar to that obtained by adenylylation.

Effect of Nitration on the Affinity of GS for ADP. The unadenylylated form of GS has a higher affinity for ADP than does the adenylylated enzyme. This is illustrated by the data in Table 2, which show that the transferase activity of unadenylylated GS is about the same at both high (0.4 mM) and low (5.0 μM) concentrations of ADP, whereas the transferase activity

Table 2. Effect of nitration on the affinity of GS for ADP

Form of GS	Relative activity	
	0.4 mM ADP	5 μM ADP
Unadenylylated	100	96
Adenylylated	100	23
Nitrated unadenylylated	100	23

Except for the indicated concentrations of ADP, the transferase activity was measured at pH 7.57 in the presence of 60 mM Mg(II) and 0.04 mM Mn(II), as described in *Materials and Methods*.

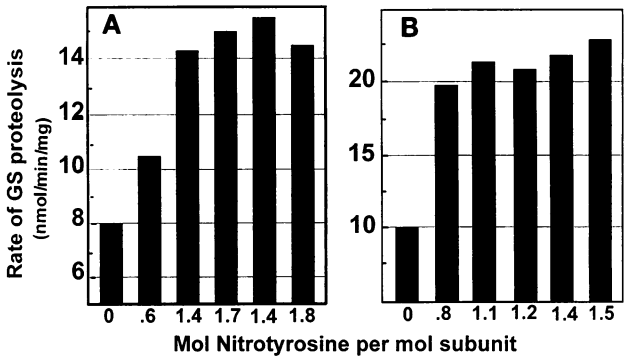

FIG. 6. Effect of nitration on susceptibility of GS to degradation by the multicatalytic protease. (*A*) Unadenylylated GS. (*B*) Adenylylated GS. Conditions were as described in Fig. 1.

of adenylylated and nitrated forms of GS at 5 μM ADP is only 25% of that obtained at the higher ADP concentration.

PN-Dependent Conversion of Methionine Residues to MXOS Residues. GS contains 16 methionine residues per subunit. When GS was exposed to PN at pH 5.5, 6.3, or 8.0, there was a PN concentration-dependent increase in the number of MSOX residues formed. It remains to be determined whether the conversion of methionine residues to MSOX contributes to the apparent increase in the state of adenylylation (see *Discussion*).

Effect of Nitration on the Susceptibility of GS to Degradation by the Multicatalytic Protease. The data summarized in Fig. 6 show that the nitration of about one tyrosine residue per subunit of either the adenylylated or unadenylylated forms of GS converts them to a form that is more susceptible to degradation by the multicatalytic protease than the unmodified forms of these enzyme species. Proteolysis of the different forms of GS (16 μM) by the multicatalytic protease (0.3 μM) was performed at 37°C in 50 mM Hepes/100 mM KCl, pH 7.8, as described in *Materials and Methods*.

DISCUSSION

Treatment of GS with PN leads to nitration of tyrosine residues and conversion of methionine residues to MSOX residues. In the absence of Fe-EDTA, the nitration of a single tyrosine per subunit of unadenylylated GS converts the enzyme to a form exhibiting regulatory characteristics similar to those obtained *in vivo* by enzyme-catalyzed adenylylation of a single tyrosine residue per subunit. In contrast, nitration of tyrosine residues in the adenylylated form of GS leads to complete inactivation of the enzyme. Nitration of 1 tyrosine residue per subunit converts both the adenylylated and unadenylylated forms of GS to forms that are susceptible to degradation catalyzed by the multicatalytic protease. It is noteworthy that in the presence of 0.1 M KCl the linear relationship between the ratio of Mg(II)/Mn(II)-supported activity extrapolates to a value of 6 nitrotyrosine residues per dodecamer rather than the expected value of 10–12. In view of the fact that GS is likely composed of six dimers arranged in a hexagonal ring (13), it is possible that KCl stabilizes the dimers such that nitration of one subunit of the pair converts both subunits in the dimer to the adenylylated configuration.

In agreement with the report of Beckman *et al.* (14), high concentrations of Fe-EDTA greatly enhance the rate and extent of tyrosine nitration, but under these conditions nitration of five to six tyrosine residues per subunit is needed to convert GS to a form that mimics the effect of adenylylation. Moreover, nitration of more than one tyrosine residue per subunit leads to a decrease in catalytic activity.

There is no consistent relationship between the PN-dependent formation of MSOX and the $\bar{n}$-app value of GS.

High concentrations of Fe-EDTA have no affect on MSOX formation. Nevertheless, further studies are needed to ascertain if MSOX formation contributes to changes in the $\bar{n}$-app.

The fact that PN promotes nitration of free tyrosine but not of *o*-phosphotyrosine is understandable, since electrophilic substitutions in the ortho position of esterified tyrosine derivatives is unfavorable. Likewise, nitration of tyrosine would be expected to inhibit phosphorylation. This may account for the fact that nitration inhibits tyrosine phosphorylation of a model peptide substrate catalyzed by the lck protein kinase (17).

Although it is generally accepted that the nitronium ion (NO_2^+) is the most likely reactive intermediate in the nitration of tyrosine, the possibility that other reactive species are involved deserves consideration. The observation that Fe-EDTA facilitates tyrosine nitration, but is without effect on the conversion of methionine to MSOX, indicates that different reactive species are implicated in these two processes. Pryor *et al.* (15) have presented evidence suggesting that HOONO is converted to a more reactive species, HONOO*, that can react with methionine residues by a two-electron oxidation mechanism. With the recent discovery that $OONO^-$ reacts with CO_2 to form the putative highly reactive intermediate, $OONOCO_2^-$, which can nitrate tyrosine (16), a role of CO_2 in these reactions must also be considered. Results of our preliminary studies have established that nitration of tyrosine residues in GS is in fact greatly stimulated by the presence of CO_2.

In any case, in view of the facts (*i*) that nitration of tyrosine residues is an irreversible process, (*ii*) that nitration of tyrosine residues in GS mimics the effects of adenylylation of the enzyme, and (*iii*) that nitration prevents the phosphorylation of tyrosine residues by protein kinases, it is obvious that the regulatory function of proteins that undergo phosphorylation or adenylylation of tyrosine residues in signal transduction cascades might be seriously compromised by PN-promoted nitration.

1. Stadtman, E. R. & Ginsburg, A. (1974) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), Vol. 10, 3rd Ed., pp. 755–807.
2. Stadtman, E. R. (1991) *The Proceedings of the Robert A. Welch Research Foundation Conference on Chemical Research: XXXV Chemistry at the Frontiers of Medicine* (Welch Foundation, Houston), Vol. 35, pp. 182–203.
3. Cimino, F., Anderson, W. B. & Stadtman, E. R. (1970) *Proc. Natl. Acad. Sci. USA* **66,** 564–571.
4. Ischiropoulos, H., Zhu, L., Chen, J., Tsai, J.-H. M., Martin, J. C., Smith, C. D. & Beckman, J. S. (1992) *Arch. Biochem. Biophys.* **298,** 431–437.
5. Ischiropoulos, H. & Al-Mehdi, A. B. (1995) *FEBS Lett.* **364,** 279–282.
6. Miller, R. E., Shelton, E. & Stadtman, E. R. (1974) *Arch. Biochem. Biophys.* **163,** 155–171.
7. Mohr, S., Stamler, J. S. & Brüne, B. (1994) *FEBS Lett.* **348,** 223–227.
8. Pryor, W. A., Cueto, R., Jin, X., Koppenol, W. H., Neu-Schwemlein, M., Squadrito, G. L., Uppu, P. L. & Uppu, R. M. (1995) *Free Radical Biol. Med.* **18,** 75–83.
9. Stadtman, E. R., Smyrniotis, P. Z., Davis, J. N. & Wittenberger, M. E. (1979) *Anal. Biochem.* **95,** 275–285.
10. Rivett, A. J. & Levine, R. L. (1990) *Arch. Biochem. Biophys.* **278,** 26–34.
11. Schecter, Y., Burstein, Y. & Patchornik, A. (1975) *Biochemistry* **14,** 4497–4503.
12. Steers, E., Jr., Craven, G. R., Anfinsen, C. B. & Bethume, J. L. (1965) *J. Biol. Chem.* **240,** 2478–2484.
13. Maurizi, M. R. & Ginsburg, A. (1985) *Curr. Topics Cell. Regul.* **26,** 191–205.
14. Beckman, J. S., Ischiropoulos, H., Zhu, L., Van Der Woerd, M., Smith, C., Chen, J., Harrison, J., Martin, J. C. & Tsai, M. (1992) *Arch. Biochem. Biophys.* **298,** 438–445.
15. Pryor, W. A., Jin, X. & Squadrito, G. L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11173–11177.
16. Sergei, V. & Hurst, J. K. (1995) *J. Am. Chem. Soc.* **117,** 8867–8868.
17. Kong, S.-K., Yim, M. B., Stadtman, E. R. & Chock, P. B. (1996) *Proc. Natl. Acad. Sci. USA* **93,** in press.
18. Friguet, B., Szweda, L. I. & Stadtman, E. R. (1994) *Arch. Biochem. Biophys.* **311,** 168–173.