Oxidative Modification of *Escherichia coli* Glutamine Synthetase

DECREASES IN THE THERMODYNAMIC STABILITY OF PROTEIN STRUCTURE AND SPECIFIC CHANGES IN THE ACTIVE SITE CONFORMATION*

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Metal catalyzed oxidation of specific amino acid residues has been proposed to be an important physiological mechanism of marking proteins for proteolytic degradation. After initial oxidative inactivation of dodecameric *Escherichia coli* glutamine synthetase (GS), the integrity of the GS active site and protein structure was assessed by monitoring ATP binding, observing a susceptibility of GS to tryptic cleavage, and comparative thermodynamic analysis. The tryptic cleavage rates of an active site linked central loop were significantly accelerated for the oxidized conformer. This tryptic cleavage was essentially prevented in the presence of glutamate for native GS but not for the oxidized conformer. The integrity of the ATP binding site in the oxidized GS was substantially altered as indicated by the reduction in fluorescence enhancement associated with ATP binding. Decreases in the free energies of quaternary protein structure and subunit interactions due to oxidative modification were determined by temperature and urea induced unfolding equilibrium measurements. Comparative thermal stability measurements of a partial unfolding transition indicated that the loss in stabilization free energy for the oxidized GS conformer was 1.3 kcal/mol dodecamer. Under alkaline conditions, the urea-induced disruption of quaternary and tertiary structures of oxidized and native GS were examined. This comparative analysis revealed that the free energies of the subunit interactions and unfolding of the dissociated monomers for oxidized GS were decreased by 1.5 and 1.7 kcal/mol, respectively. Our results suggest that small free energy decreases in GS protein structural stability of only 1–2 kcal/mol may be responsible for the selective proteolytic turnover of the oxidized GS.

Protein degradation in *vivo* is a fundamental biological event yet the specific structural factors which render proteins susceptible to proteolytic clearance are not resolved (1). Proteins become substrates for proteolytic attack either through inherent structural determinates or alterations in structure as a result of covalent modification. One covalent modification reaction that has received increasing attention is oxidation of proteins by activated oxygen species (*i.e.* O$_2^\cdot$, H$_2$O$_2$, OH$^-$ (2, 3)). Activated oxygen species are generated as a result of the reduction of oxygen by electron transfer and other redox active proteins. The requirement and induction of activated oxygen scavenger enzymes such as superoxide dismutase and catalase in response to oxidative stress emphasizes the biological importance of protection against oxygen free radical damage (4, 5).

The deleterious effects of activated oxygen reactions with proteins can occur through nonspecific (global) or specific (localized) mechanisms. Nonspecific damage can be simulated by generating activated oxygen species in situ from radiation sources ($^{60}$Co) or pulse radiolysis techniques (3). Generally, this type of oxygen-induced damage is not a site-specific event, although amino acids such as Tyr, Trp, Met, His, and Cys are more susceptible to radiation mediated oxidative damage. This type of damage is caused by the generation of activated oxygen species which diffuse through the solution and react nonspecifically with the protein. Localized damage can occur when activated oxygen species such as hydroxyl radicals are formed at putative metal binding sites in proteins (2). The metals iron or copper can replace the intrinsic metals such as Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$, Zn$^{2+}$, or are themselves natural metal cofactors. When these sites are occupied by iron or copper, they can, in the presence of suitable reductants, react with oxygen via a modified Haber-Weiss mechanism to generate activated oxygen species (OH•, ferryl ion); the activated oxygen will react preferentially with amino acid residues in the vicinity of the metal binding site. Both global and localized oxidative damage to proteins results in their increased susceptibility to proteolytic attack (6, 7).

At present, the *in vivo* frequency of this oxidative event and proportions of proteins which undergo oxidative covalent modification preceding proteolytic degradation are unknown. However, the fact that oxidative modification of proteins (enzymes) renders them highly susceptible to proteolytic degradation by endogenous proteases and by purified preparations of various proteases, *in vitro*, is well documented (2).

The enzyme glutamine synthetase (EC 6.3.1.2), which catalyzes the reaction,

\[
\text{Glutamate} + \text{NH}_4^+ + \text{ATP} \rightleftharpoons \text{ADP} + \text{P}_i + \text{glutamine},
\]

is susceptible to iron-catalyzed oxidative damage. This is a key enzyme for nitrogen assimilation and subject to exquisite metabolic control in *Escherichia coli* (8). The 622,000-Da enzyme from *E. coli* is composed of 12 homologous subunits, and the active site is located at heterologous subunit interfaces. The enzyme possesses two metal binding sites for the physiological metal ion Mg$^{2+}$ (termed *n*$_1$ and *n*$_2$) that are located in the active site. These metal ions are absolutely required for activity expression. In addition, GS* can bind a

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1 The abbreviations used are: GS, glutamine synthetase, Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, MCO, metal catalyzed oxidation; SDS, sodium dodecyl sulfate.

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variety of divalent transition metal ions (9, 10). Earlier observations in this laboratory support the view that the rapid clearance of glutamine synthetase which occurs in response to changes in nitrogen metabolism involves the iron/oxygen-dependent inactivation of the enzyme prior to its proteolytic degradation. Moreover, inactivation and degradation of GS is dramatically increased in cells which are deficient in catalase (11). Inactivation of GS in vitro by metal catalyzed oxidation (MCO) is associated with the covalent modification of at least two amino acids (His and Arg) located at the low affinity metal binding site, $n_H$ (12-14). The localized nature of the MCO of GS is illustrated by the observation that the transition state analog enzyme complex consisting of L-methionine sulfoxide phosphate-metal-GS is completely protected against oxidative inactivation.8

In the present study, conformational alterations of GS resulting from iron catalyzed oxidative modification at the active site were examined by monitoring changes in the rates of cleavage of an active site linked central loop by trypsin and by the tryptophan fluorescence enhancement induced by the binding of ATP. The protein stability changes were determined using temperature and urea as physical perturbers of protein structure.

**MATERIALS AND METHODS**

**Chemicals**—Ascorbate was obtained from Aldrich chemical company. Trypsin and glutamate were purchased from Sigma. Desferroxamine was provided by CIBA Pharmaceutical Co. Urea was purchased from Baker Chemical company. ATP was purchased from Fluka. Chelex 100 resin (200-400 mesh) was obtained from Sigma and was prepared as described previously (15).

**Purification of GS**—Glutamine synthetase was isolated from E. coli (YMC10/1p9ml) which overproduces the enzyme. GS was purified using the zinc-induced aggregation method (16) followed by ammonium sulfate and acetone treatment (17). Protein concentrations were determined by absorbance measurements at 290 nm using $A_{280}$ of a 0.1% solution (1 cm) = 0.387 (18). Activity measurements were performed using the glutamyl transferase assay method (19). The GS preparations used in these experiments contained approximately 1.5–0.7 adenylated subunits/dodecamer and possessed transferase activities of 120–130 units/mg.

**Iron Catalyzed Oxidation of GS**—Unless specified, the initial buffer system used in all experiments contained 50 mM Heps, 100 mM KCl, 10 mM MgCl$_2$, pH 7.4, 25°C (referred throughout this paper as “standard buffer”). Iron-catalyzed oxidation of GS was obtained by incubating GS (1 mg/ml) in the standard buffer supplemented with 5–50 $\mu$M FeCl$_3$ and 20 mM ascorbate at 37°C. Enzyme inactivation was monitored by the time-dependent loss of glutamyl transferase activity. With remaining activity $r$ of the original value, the oxidation reaction was stopped by the addition of 0.1 volume of a stock solution of metal chelators containing 100 mM inorganic pyrophosphate, 10 mM EDTA, and 10 mM desferroxamine. This mixture was allowed to equilibrate for 10 min. An absorption spectrum was measured in a stirred cuvette to give final concentrations of 0.6–1.1 $\mu$M GS and 0.8–1.5 $\mu$M trypsin in a buffer containing 50 mM Heps, 100 KCl, 1 mM MnCl$_2$, pH 7.4, at 37°C. The molar ratio of GS to trypsin was maintained at 1:1.3. An equal aliquot of trypsin was added to the reference cuvette prior to each kinetic run. The spectral contributions due to trypsin were separately determined. This is illustrated in the observation that the second derivative $r$ value for either oxidized or native GS remained unchanged before and after trypsin addition. In the absence of added substrates for GS, spectra were collected every 10 s with 5-s integration times (10 spectra/measurement) and stored on disk for later analysis. The second derivative $r$ value was calculated at each time point, plotted as a function of time, and the data were fit as discussed under “Results.” Since trypsin oxidation of GS was significantly slower in the presence of 0.85 mM ATP and 100 mM glutamate, in studies with these GS substrates, spectra were collected every 30 s with 5-s integration.

The time-dependent trypsin-induced fragmentation of GS was analyzed as follows: at indicated times during the kinetic run, 10–$\mu$M aliquots of the reaction mixture were mixed with an equal volume of 50 mM Tris-HCl, pH 8.4, 9% SDS, 25 $\mu$M phenylmethylsulfonyl fluoride and boiled for 2 min at 95°C. After the kinetic run was complete, the individual samples were loaded onto a pre-cast Pharmacia 5–25% acrylamide gradient Phast gel (9 g of protein per lane) and electrophoresed using precast SDS buffer strips on a Pharmacia Phast System. Protein was visualized by Coomassie Blue staining.

**ATP Binding Measurements**—Steady state fluorescence spectra were recorded with a SLM 4800 spectrophotometer equipped with a dedicated temperature controller (model 89100A) and an attached pelier junction temperature-controlled cuvette holder (model 89101A) with a built-in magnetic stirrer and temperature probe.

**Tryptsin Proteolysis**—Kinetics of GS cleavage by trypsin were followed by monitoring the time dependent changes in tyrosine solubilization. The reaction was initiated by rapid dilution and mixing of a concentrated stock of trypsin (1 mg/ml) in a stirred cuvette to give initial concentrations of 0.6–1.1 $\mu$M GS and 0.8–1.5 $\mu$M trypsin in a buffer containing 50 mM Heps, 100 KCl, 1 mM MnCl$_2$, pH 7.4, at 37°C. The molar ratio of GS to trypsin was maintained at 1:1.3. An equal aliquot of trypsin was added to the reference cuvette prior to each kinetic run. The spectral contributions due to trypsin were separately determined. This is illustrated in the observation that the second derivative $r$ value for either oxidized or native GS remained unchanged before and after trypsin addition. In the absence of added substrates for GS, spectra were collected every 10 s with 5-s integration times (10 spectra/measurement) and stored on disk for later analysis. The second derivative $r$ value was calculated at each time point, plotted as a function of time, and the data were fit as discussed under “Results.” Since trypsin oxidation of GS was significantly slower in the presence of 0.85 mM ATP and 100 mM glutamate, in studies with these GS substrates, spectra were collected every 30 s with 5-s integration.

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**Thermal Analysis**—The thermal analysis was performed by using a Hewlett-Packard model 8450A rapid scan diode array spectrophotometer with a dedicated temperature controller (model 89100A) and an attached pelier junction temperature-controlled cuvette holder (model 89101A) with a built-in magnetic stirrer and temperature probe.

The metal-dependent reversible thermal transition was examined as follows: the reference cuvette contained standard buffer and was maintained at 30°C. The instrument was programmed to increase the temperature in 1°C increments in the transition region with 7-min equilibration time between absorption measurements. Independent measurements on similar samples indicated that the thermal transition was complete within 1 min. After equilibration, 120 absorption spectra were collected at two spectra/s. The final collected absorption and second derivative spectra represent an average of 120 spectra. After the thermal transition was complete, the protein solution was cooled to the original starting temperature (30°C) and allowed to equilibrate for 15 min. An absorption spectrum was measured to confirm reversibility.

The overall thermal stability of GS was assessed by examining the irreversible temperature-induced aggregation reaction of GS. The temperature-dependent aggregation was monitored by the increased light scattering at 320 nm. Briefly, 0.3 $\mu$M GS (dodecamer) was

9 R. L. Levine, personal communication.
initially equilibrated at 50 °C for 10 min in the standard buffer. The thermally induced absorbance changes were recorded as a function of time as the temperature was increased at a programmed rate of 1 °C/min from 50 to 80 °C in a stirred cuvette. The time-dependent absorbance changes were stored and analyzed by converting the time-based measurements to °C (1 °C/min). Upon return to 30 °C the absorbance remained at the high temperature limit, and the protein was inactive as assessed by the transferase assay. This indicates that the heat denaturation was an irreversible reaction.

Urea Unfolding Experiments—The urea-induced dissociation and unfolding profile of oxidized and native GS was monitored by observing changes in tyrosine solvation by the second derivative analysis outlined above. Since each subunit contains 17 tyrosine residues, this spectroscopic technique was assumed to be inherently more sensitive to structural changes due to subunit dissociation and unfolding than would monitoring local changes of the two tryptophan residues. The tryptophan residues are located exclusively on the amino-terminal portion of the molecule, and local solvation changes surrounding these tryptophan microenvironments might not reflect global structural changes. A 10-μl aliquot of concentrated GS (10 mg/ml) was diluted into a sealed Eppendorf vial containing 0.5 ml of buffer composed of 50 mM Tris-HCl, 100 mM KCl, 10 mM MgCl₂, pH 8.3, containing a given amount of urea. The final concentration of GS per sample was 0.5 mg/ml. The sealed samples were incubated at 20 °C for 1-2 days. The system was assumed to have reached equilibrium, because no further spectral changes occurred after this time period.

At each urea concentration sample, a total of 180 spectra were collected at two spectra/s to generate one averaged spectrum. Data collection was repeated six times, and the reported r value at each urea concentration is the mean of six averaged spectra.

RESULTS

Trypsin Cleavage of the Active Site-linked Central Loop—GS is susceptible to proteolytic cleavage by a number of secreted proteases such as subtilisin, trypsin, chymotrypsin, and V8 protease (25, 26). Initial proteolysis by these proteases results in a specific cleavage of a central loop region in GS which is located in the central core of the dodecamer (27, 28). Although the clipped enzyme shows significantly lower activity, the protein retains its dodecameric structure after this initial cleavage event. X-ray crystallographic analysis has revealed that this central loop (residues 158-188) is directly linked to the active site (14). In addition to the crystallographic data, experiments have shown that this loop region was protected against trypsinic cleavage in the presence of glutamate (26). Also, covalent modification of Arg-172 by ADP-ribosylation (29) inactivates GS. Based on these observations, Eisenberg and co-workers (14, 28) have suggested that movements of this loop region are directly linked to the active site (14). Since this central loop region is linked to the active site, the susceptibility of this region to proteolytic cleavage could be affected by oxidative modification at the metal cation binding sites. Molecular modeling studies have revealed that trypsin can fit into the central channel where the subunit central loop resides (14). In the present study, it was found that under these conditions changes in GS protein structure resulting from trypsin proteolysis of this central loop could be easily monitored spectrophotometrically by following changes in the tryrosine-sensitive uv second derivative r value (Figs. 1A and 3, A and B).

The kinetic profiles of the second derivative signal changes show distinct lag phases followed by a more rapid reaction for both the oxidized and native GS conformers. Since these profiles are reminiscent of consecutive first order reactions, the trypsin-dependent second derivative r value changes were fit to a consecutive first order model,

\[ k_1 \rightarrow k_2 \rightarrow N \rightarrow N^* \rightarrow N^{**} \]

where N is the initial protein, and N* and N** are trypsin-altered conformers of GS. The kinetic profiles were fit using a nonlinear least square program to the equation,

\[ r_{obs} = \Delta r_{max} + (-1/(k_1 - k_2))(k_2 \exp^{-kt} - k_1 \exp^{-kt}) + r_0 \]

where \( r_{obs}, \Delta r_{max} \), and \( r_0 \) are the observed, maximal amplitude, and the initial r values, respectively. The residual plots (Fig. 1B) show no significant deviations in relation to the fit line (Fig. 1A). The calculated rate constants (\( k_1 \) and \( k_2 \)) are listed in Table I. The initial rate, \( k_1 \), was found to be similar for both the oxidized and native conformers, whereas \( k_2 \) increases approximately 5-fold for oxidized GS in comparison with the native form.

In the presence of the substrates, glutamate (100 mM) or glutamate plus ATP (0.85 mM), the differences in proteolysis rates between the oxidized and native conformers were substantial. The trypsin-induced second derivative r value of native GS is characterized by rather small changes, whereas the oxidized conformer exhibits large changes in the r value in the same time frame (Fig. 2). In the absence of substrates (ATP and glutamate), the native and oxidized forms of GS are both almost completely cleaved during 2-h incubations with trypsin (Fig. 3C, lanes 3 and 4). However, in the presence of substrates, the native enzyme is almost completely resistant to cleavage by trypsin, whereas the oxidized enzyme is still readily cleaved (Fig. 3C, lanes 5 and 6). There is some substrate protection of oxidized GS against trypsin proteolysis in either the presence of glutamate or glutamate + ATP as...
TABLE I

<table>
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<tr>
<th>Ligands</th>
<th>Rates × 10^{-3}/s</th>
<th>$k_1$</th>
<th>$k_2$</th>
</tr>
</thead>
<tbody>
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<td>Native</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>2.5 ± 0.5</td>
<td>2.4 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Oxidized</td>
<td>2.8 ± 0.8</td>
<td>9.3 ± 0.9</td>
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</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>3.7 ± 0.5</td>
<td>4.2 ± 0.7</td>
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<td>Mg$^{2+}$ + Glu$^b$</td>
<td>2.4 ± 0.4</td>
<td>4.2 ± 0.1</td>
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</table>

A. $A = B = C$. A consecutive first order reaction model provided a good fit to the data. The observable increase in the second derivative $r$ value is assumed to denote the time-dependent increase in the trypsin clipped GS and is represented by species C in the equation.

$^b$The concentrations of glutamate (Glu) and ATP were 100 and 0.85 mM, respectively. Each measured value represents the mean and S.D. of a minimum of three measurements.

Fig. 2. Trypsin-induced second derivative $r$ value changes of oxidized and native GS in the presence of 100 mM glutamate and 0.85 mM ATP. Buffer conditions are the same as described in the Fig. 1 legend. In the presence of trypsin, the oxidized conformer shows a significant time-dependent spectral change. The native enzyme shows minor spectral changes during the same incubation period.

Fig. 3. SDS-gel electrophoresis was used to analyze the tryptic fragmentation patterns of native (gel A) and oxidized (gel B) GS samples taken (lanes 1–8) at 0, 0.5, 2, 4, 6, 10, 15, and 24 min, respectively, during the trypsin cleavage reaction profile illustrated in Fig. 1. The samples (lanes 1–6) applied to the final gel (gel C) are molecular weight markers (lane I), native GS (lane 2), and trypsin-cleaved native and oxidized GS (lanes 3 and 4). The resulting fragments from limited trypsin digestion for both species are the same. In lanes 5 and 6, native and oxidized GS were exposed to trypsin in the presence of glutamate (100 mM) for 3 h. The data indicate that glutamate binding to the oxidized conformer no longer protects this species from trypsin cleavage.

Fig. 4. The extent of ATP-induced fluorescence enhancement of tryptophan emission intensity at 20 °C upon excitation at 300 nm for native and oxidized GS. The concentrations of GS and ATP used in these experiments were 0.7 μM and 2.5 mM, respectively. No significant fluorescence changes were observed after further additions of ATP (above 2.5 mM). The fluorescence spectra for native and oxidized GS in the presence of 10 mM MgCl$_2$ shows the loss of tryptophan-dependent fluorescence enhancement when ATP is added to the oxidized conformer.

Evidenced by the decreases in the calculated rate constants $k_1$ and $k_2$ (Table I). However, this protective effect is substantially less than that observed for the native conformer. Normalized signal changes for the trypsic cleavage of the native protein in the presence of glutamate + ATP or glutamate are the same (data not shown). This suggests that ATP binding does not play a substantial role in altering the exposure of the active site-linked central loop to trypsic cleavage. In fact, ATP addition alone does not inhibit the observed proteolysis rates of both the oxidized and native conformers (data not shown). In agreement with previous results (26), proper glutamate binding at the active site is responsible for the decreased trypsic cleavage at the central loop region of native GS.

The higher initial $r$ values of the oxidized conformer are always observed and are presumably due to the increased solvation of 1 or more tyrosine residues. In the absence of trypsin, the higher initial $r$ values observed after ATP addition to either oxidized or native GS could be due to: 1) ATP absorption interferes with the original aromatic amino acid spectrum, 2) conformational changes induced by ATP binding, or 3) a combination of both ATP absorption and conformational changes. It is noteworthy that the final second derivative spectrum and corresponding $r$ value for the trypsin-cleaved central loop GS conformers are the same as those observed for the metal-free (apo) GS conformer.

Oxidative Alteration of the ATP Binding Site—It was shown previously that the binding of ATP to the catalytic site of native GS is associated with a substantial increase in tryptophan fluorescence. Therefore, the integrity of the active site of oxidized GS was probed by monitoring changes in tryptophan fluorescence intensity in the presence of ATP. At concentrations of ATP sufficient to saturate the active site of the native conformer, oxidized GS exhibits only a very small increase in tryptophan fluorescence intensity, whereas a 2-fold increase is observed with native GS (Fig. 4). These data indicate that oxidative modification results in the loss of integrity of the nucleotide binding site. The calculated dissociation constant, $K_d$, obtained from ATP titrations with native GS outlined in this paper was 188 μM. This value is similar to the previously measured dissociation constant (260 μM) obtained earlier (30).

Thermal Stability of Oxidized GS—GS exhibits a metal-dependent (Mn$^{2+}$ or Mg$^{2+}$) reversible thermal transition which can be monitored by temperature-dependent solvation changes in tyrosine and tryptophan. Although tyrosine-
tryptophan-dependent thermal transition profiles can be fit by a simple two-state model, the transitions are nonidentical (31). This observation indicates that a more complex reaction is occurring. The existence of separate thermal transitions for these intrinsic chromophores could indicate that changes in these signals reflect different regions of the protein (31). Light scattering measurements indicate that no major conformational transitions, such as aggregation or subunit dissociation, accompany the thermal transition. By means of differential scanning calorimetry evidence was obtained that these spectrally observed thermal transitions may reflect cooperative transitions (32). Furthermore, these transitions likely involve the partial unfolding of the entire dodecameric structure and not that of the individual subunits. In relation to the total protein present, the differential scanning calorimetry-derived enthalpies are smaller than expected for a global denaturation reaction.

The source of the reversible thermally induced uv absorption changes is not known at the molecular level. From an examination of the x-ray crystallographic data, it seems possible that those regions of the enzyme that are involved in the temperature-induced metal-dependent partial unfolding reactions included the tyrosine rich carboxyl-terminal domain which contains 15 of the 17 Tyr residues per subunit and the tryptophan-containing amino-terminal domain of the heterologous subunit active site interface (26). If this is true, then the observed thermal transition would be affected by localized iron-catalyzed oxidation of amino acids in the active site of GS. To investigate this possibility, the thermally induced partial unfolding transitions of oxidatively modified and native GS conformers were examined and compared. Since the carboxyl-terminal domain provides all the necessary ligands for the specific metal binding sites at the active site interface, the tryptophane-sensitive thermal transition profiles only were examined in the experiments outlined below.

The thermally induced changes in the tyrosine-sensitive r value for native and oxidized GS appear to follow a cooperative transition (Fig. 5A). The pre- and post-denaturation base lines were determined by a linear least squares analysis. The thermal equilibrium constant for the partial unfolding reaction \( N \rightleftharpoons N^* \), \( K_m \) at each temperature, \( T \), was determined by the relationship

\[
K_m(T) = \frac{r(T) - r_o(T)}{r(T) - r(T^*)}
\]

where \( r(T) \) is the second derivative r value at temperature \( T \), \( r_o(T) \) and \( r(T^*) \) are the second derivative values at temperature, \( T \), derived from the extrapolated base lines. The van’t Hoff enthalpy \( \Delta H_{vH} \), transition temperature \( T_m \) and \( r_{max} \) were obtained from the following equations.

\[
K_m(T) = \exp[-\Delta H_{vH}(1/T - 1/T_m)/R]
\]

\[
\Delta r_{calc} = \Delta r_{max} \cdot K_m(T)/(1 + K_m(T))
\]

The \( \Delta r \) values (after subtraction of the extrapolated base lines) were fit with the corresponding \( \Delta r_{calc} \) values by the adjustment of the above indicated parameters with a nonlinear least squares program provided by Dr. Andrew Shrake. Best fits were obtained by minimizing the sum of the square of residuals.

A comparison of the tyrosine-dependent two-state van’t Hoff thermal transitions (Fig. 5B) indicates that the thermally induced native conformer profile occurs at higher temperatures and is more cooperative than is the profile of oxidized GS. Oxidation of GS is accompanied by a decrease in the transition temperature \( T_m \) of 3.4 °C and a decrease in enthalpy by 54 kcal/mol in the presence of 10 mM Mg\(^{2+}\) (Table II). The difference in the free energy change for the thermally induced partial unfolding reaction of oxidized and native GS at the native GS \( T_m \), \( \Delta AG \), can be obtained using the relationship,

\[
\Delta AG = \Delta T_m \cdot \Delta S_m
\]

where \( \Delta T_m \) is the \( T_m \) difference between oxidized and native GS and \( \Delta S_m \) is the entropy change for native GS (33). Given the above equation, when \( \Delta T_m = 3.4 \) °C and \( \Delta S_m = 322 \) cal/deg mol, then \( \Delta AG = 1.3 \) kcal/mol (Table III). Since the thermally induced partial unfolding reaction is a function of the entire dodecamer, then this free energy change reflects the loss in stabilization free energy per dodecameric unit.

The effects of added substrates such as ATP and glutamate on the thermally induced partial unfolding reaction was also examined for both native and oxidized GS. For both protein species, substrate addition resulted in increases in the observed transition temperatures (Table II). Note, however, that differences between the \( T_m \) observed in the absence (+10 mM Mg\(^{2+}\) only) and presence of substrates (+0.9 mM ATP or 100 mM glutamate) were smaller for the oxidized conformer (2–3 °C) than for the native GS (4–7 °C). In addition, the loss in

![Fig. 5. A, temperature-dependent changes in the second derivative r value for native (■) and oxidized (○) GS in the temperature range of 30–62 °C. B, the bottom panel represents a simple two-state fit to the observed spectral data for each of the above GS species. The calculated enthalpy and \( T_m \) values for native and oxidized GS were 124 kcal/mol, 53.1 °C, and 78 kcal/mol, 50.7 °C, respectively.](image-url)
Tbenn between native and oxidized native GS. This could indicate a loss in cooperativity or the temperatures than does the native conformer. The temperature transition was complete. The corrected thermal transition was complete.

To determine if the partial unfolding reaction reflects the overall thermal stability of the protein, the temperature dependence of the irreversibly thermally induced aggregation of the oxidized and native conformers was examined. This aggregation event was monitored by the temperature-dependent increase in absorbance at 320 nm due to increased light scattering (Fig. 6) at a constant temperature increase of 1 °C/min. This experiment clearly indicates that the oxidized conformer undergoes temperature-dependent aggregation at lower temperatures than does the native conformer. The temperature where half the population has aggregated (Td) is 65.6 °C for the oxidized and native GS species, respectively. As was observed for the thermally induced partial unfolding reaction, the differences in Td between the oxidized and native GS conformers is also 3.5 °C. In addition, the breadth of the transition for the native species is sharper than that of the oxidized GS. This could indicate a loss in cooperativity or the existence of heterogenous oxidized GS conformers. These irreversible thermally induced aggregation data support the idea that the partial unfolding reaction directly reflects the overall thermal stability of the entire protein structure.

Dissociation and Unfolding of GS by Urea—The alkaline-induced urea denaturation of GS was followed by observing the urea-dependent changes in the second derivative r values (Fig. 7A). The pre- and post-denaturation base lines were determined by a linear least squares method. The corrected urea-dependent fractional changes show a clear inflexion point for both the oxidized and native GS conformers. The observed fractional changes were fit to a three-state model described by two equilibrium constants. This model can be written as follows,

\[ K_1 = \frac{N}{I} = D \]

where N, I, and D represent native, intermediate and denatured GS conformers. The apparent equilibrium constant, \( K_{app} \), was calculated by the equation given below (35),

\[ K_{app} = \frac{K_1 \cdot K_2 + Z \cdot K_1}{\left[1 + (1 - Z) \cdot K_2 \right]} \]  

(6)

where \( K_1 = [I]/[N] \) and \( K_2 = [D]/[I] \) at a given urea concentration and \( Z = (r_1 - r_N)/(r_D - r_N) \). \( r_2 \) is the second derivative ratio for the intermediate. The equilibrium constants at zero denaturant, \( K_1(H_2O) \) and \( K_2(H_2O) \) were used as adjustable parameters to estimate the urea-dependent equilibrium constants, \( K_1 \) and \( K_2 \), where \( \ln(K_{1(urea)})_{app} = \ln(K_{1(urea)}(H_2O)) + (m/RT) \cdot [urea] \). These estimated equilibria constants and m values were then used to calculate the urea-dependent \( K_{app} \) (Equation 6) for a given Z value. The urea-dependent fractional change in the r value was calculated using the following equation

\[ F_{app} = \frac{K_{app} \cdot (1 + K_{app})}{K_{app} + 1} \]  

(7)

where \( F_{app} \) is the apparent fractional change. An iterative fitting program was employed where the best estimates of \( K_1 \) and \( K_2 \) m and Z were evaluated on the basis of minimizing the sum of the squares of the residuals between the calculated \( F_{app} \) and the observed fractional change. The best estimated free energies of these transitions, derived from the equilibria constants, \( K_1(H_2O) \) and \( K_2(H_2O) \), where \( DG = -RT \ln K \) and the best m and Z value(s) are listed in Table IV. These parameters gave the best fit to the data (Fig. 7B). It should

<table>
<thead>
<tr>
<th>Ligands</th>
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<th>( \Delta S_m )</th>
<th>( \Delta G_{(native-oxidized)} )</th>
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<td>Mg(^{2+})</td>
<td>3.7</td>
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</tr>
<tr>
<td>Mg(^{2+}) + ATP(^{3-})</td>
<td>5.9</td>
<td>351</td>
<td>-2.1</td>
</tr>
<tr>
<td>Mg(^{2+}) + Glu(^{+})</td>
<td>7.8</td>
<td>420</td>
<td>-3.2</td>
</tr>
</tbody>
</table>

* ATP and glutamate (Glu) concentrations were 0.85 and 100 mM, respectively.
be pointed out that similar transitions have also been reported by Wedler and co-workers (34) for a series of thermophilic glutamine synthetases using guanidine HCl as a denaturant.

This urea-induced unfolding transition has been demonstrated to be a reversible reaction (36, 37). At low protein concentrations, at least 80–90% of the original activity can be recovered upon diluting the unfolded protein into renaturing solution conditions. At higher protein concentrations, the restoration of activity is not as extensive (60%). The decrease in recovery is due to competing nonproductive aggregation reactions.

The urea-dependent $r$ value denaturation profile of E. coli glutamine synthetase can be described by at least two equilibrium constants. Based on accumulated experimental data, the nature of the molecular reactions contributing to each equilibrium constant are proposed to be described as an oligomer ⇔ monomer ⇔ unfolded monomer reaction. When GS is incubated in a solution containing 1 mM EDTA, 1 M urea, 50 mM Tris-HCl, pH 8.7, dodecameric GS dissociated into partially folded monomers (38). Under these conditions, the presence of monomers was confirmed by equilibrium sedimentation, native gel electrophoresis, gel chromatography, and later by glutaraldehyde cross-linking experiments (37–39). Circular dichroism measurements revealed that these monomers existed as a partially folded protein as evidenced by the residual $\beta$ secondary structure (39). For both the oxidized and native GS, the second derivative spectral fingerprint of the GS species observed at the inflection points ($F_{\text{mp}} = 0.72$) in the urea-unfolding equilibrium profile is identical to the spectrum observed for the previously characterized partially folded GS monomers. Further addition of urea to this intermediate GS species resulted in an increase in the solvation of tyrosine residues as indicated by the increase in the $r$ value. It is worth noting that no changes in light scattering measurements were observed as the unfolding reaction progressed past the inflection point (data not shown). This is consistent with the hypothesis that a monomer-unfolding reaction contributes to the spectral changes above the inflection point.

Comparative analysis of the unfolding energetics for oxidized and native GS revealed that the differences in derived free energies ($\Delta G$) for each equilibrium reaction were in the range of 1.5–2 kcal/mol (Table IV). These free energy comparisons were made for these dissociation and unfolding reactions at 20°C under alkaline conditions and might not apply if the unfolding were performed under more physiological conditions. Unfortunately, at neutral pH and 25°C, GS forms insoluble aggregates in the transition zone of the unfolding reaction. However, earlier kinetic inactivation experiments with partial oxidatively inactivated GS indicated that, in the presence of 4 M urea at pH 7.15, the loss of activity was faster for the hybrid molecules (both containing oxidized and native monomers) than was observed for the native conformer (40). This observation suggested that subunit interactions were weaker for the hybrid oligomers. The results presented here suggest that the free energy differences between these two GS conformers are relatively small compared with the total free energy of dissociation and monomer unfolding.

**TABLE IV**

| Parameters for fitting the observed equilibrium transition using a three-state model |
|---------------------------------|-----------------|-----------------|
| $\Delta G_1(H_2O)$ | $\Delta G_2(H_2O)$ | $m_1$ | $m_2$ |
| Native | 6.3 ± 0.2 | 9.6 ± 0.1 | 3.86 | 2.74 |
| Oxidized | 4.8 ± 0.4 | 7.9 ± 0.4 | 2.96 | 3.64 |

and the apparent fractional change is derived from $K_{\text{app}} = (K_1K_2 + ZK_3)/(1 + (1 - Z)K_1)$. The $Z$ value that gave the best fit (minimized sum of the squares of the residuals) was 0.72.

**DISCUSSION**

Iron-catalyzed oxidation of amino acid residues located at the metal ion binding sites of E. coli glutamine synthetase has been proposed to be one mechanism by which the enzyme is marked for proteolytic clearance. In the absence of most substrates, this modification has been demonstrated to proceed rapidly (41). Results presented herein demonstrate that this localized modification alters the binding characteristics of the enzyme for glutamate and ATP, e.g., substrate-induced conformational changes, particularly those characterized by ATP- and glutamate-induced tryptophan fluorescence enhancement and central loop movements, are decreased or disappeared. At the quaternary level, the predominant structural change resulting from MCO covalent modification is a decrease in the cooperativity of the subunit interactions; this translates to free energy decreases on the order of 1–2 kcal/mol.

MCO of GS results in a conformational change which renders the active site-linked central loop more susceptible to cleavage by trypsin compared to the native enzyme. The proteolytic fragments generated by trypsin cleavage are the same for the oxidized and native conformers. This suggests that the oxidation of GS does not lead to substantial unfolding or denaturation of the enzyme. The kinetics of the tryptic cleavage reaction, monitored by tyrosine-sensitive second derivative changes, are consistent with the participation of two consecutive first order reactions. In the absence of substrates, the initial rate, $k_1$, for the oxidized and native conformers is similar, whereas $k_2$ is 5-fold higher for the oxidized protein compared with the native enzyme. One possible explanation for the consecutive first order mechanism is that an initial tryptic cleavage of one loop of the dodecamer, reflected in $k_1$, would result in the enhanced exposure and tryptic cleavage of the remaining loops of the dodecamer. The accelerated cleavage rate, $k_2$, of the oxidized conformer could reflect a decreased cooperative interaction between the subunits for this species, resulting in a faster relaxation of the dodecameric structure.

Since there are a total of 17 tyrosine residues/subunit of GS, it is difficult to identify those tyrosine residues that become solvated as a result of tryptic cleavage. However, x-ray crystallographic analysis of GS shows that 2 tyrosine residues (Tyr-164 and -179) are located within the central loop (156–188) (14). Part of this central loop extends into the active site of this enzyme. The hydroxyl moiety of tyrosine 179 is 5 Å from the $\eta_1$ metal cation binding site. This invites speculation that solvation of these particular tyrosine residues contributes to the second derivative changes observed during tryptic cleavage.

The trypsin-catalyzed cleavage of native GS is much slower in the presence of the substrates glutamate or ATP plus glutamate (Fig. 2). The rates of cleavage were unaffected by the presence of ATP. This indicates that conformational changes due to glutamate binding at the active site of native GS decreases the access of the trypsin to central loop region, in agreement with earlier results (26). In contrast, the central loop of the oxidized conformer remains susceptible to tryptic
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cleavage in the presence of ATP and glutamate. This implies that the binding of these substrates to the oxidized conformer may be decreased or altered.

Our results are interpretable in the light of the structural characteristics of GS established by earlier investigations. Thus there is evidence that ATP binds to both the amino- and carboxyl-terminal domains at the heterologous subunit interface (28, 43) and that binding of the metal-ATP complex to GS results in a 2-fold enhancement of the tryptophan fluorescence (30). The low affinity site n2 is involved in the binding of the metal-ATP complex (30, 42). Moreover, Trp-57 which is located in a loop connecting two β strands of the amino-terminal domain close to the ATP binding site (43) is the source of the enhanced fluorescence (44). It is also established that the inactivation of GS by MCO systems is associated with the modification of at least 2 amino acid residues at the n2 binding site. Specifically, histidine 269, which ligates metal at the n2 site, and arginine 344, 5 Å away from the n2 site (14) are oxidized to asparagine and γ-glutamyl semialdehyde, respectively (12, 13).

In view of these results, our observation that oxidation of GS leads to a loss in the ability of ATP to enhance the tryptophan fluorescence can be attributed to an alteration in the ATP-metal binding site. Nevertheless, it is evident that the substrates, glutamate and ATP can still bind to the oxidized GS, since there is an increase in the Tm values of the reversible thermal transition when these substrates are present in the buffer. However, the substrate-induced increase in the thermal transition temperature observed with the oxidized enzyme is substantially less than that for native GS. Consequently, native GS has a higher free energy of stabilization due to the increased substrate binding free energy. This may account for the fact that substrates protect the native conformer from degradation by intrinsically proteases.

It is evident from the present work and that of earlier studies that oxidation of GS leads to a weakening of the subunit interactions, but the molecular basis for the observed changes is still obscure. A role of divalent metal ions (Mn2+ or Mg2+) in stabilization of the quaternary structure is evident from the fact that removal of divalent metals from the enzyme facilitates subunit dissociation under mild denaturing conditions (38–40) and the fact that reassociation of the subunits to generate the dodecamer does not occur in the absence of either Mn2+ or Mg2+ (36, 37). Nevertheless, according to the structure deduced from x-ray crystallographic model, the amino acid residues involved in the ligation of metals at the n1 and n2 binding sites do not reside at subunit contact sites of the dodecamer (14). It is therefore difficult to reconcile the fact that primary oxidative modification occurs at the n1 metal binding site with the fact that the oxidation has a marked effect on the stability of the quaternary structure. It is implicit that the alterations at the n2 site can trigger more generalized changes in protein conformation, encompassing the subunit-subunit interaction sites. That this is the case is supported by the recent genetic engineering studies of Villafranca and co-workers where His-269, located at the n2 site, was changed to either Asp or Asn to examine the altered activity and metal ion binding affinities. The presence of Asp or Asn at this site led to an increase or a decrease, respectively, in the metal ion binding affinities at both the n1 and n2 binding sites. The studies of Levine and co-workers (23, 46) also showed the mild oxidation of GS leads to a decrease in initial ion binding at both the n2 and n1 sites and also to an increase in hydrophilic character of the enzyme as deduced by hydrophobic chromatography.

In summary, inactivated GS, which has undergone short term metal-catalyzed oxidative damage, exhibits decreased thermal stabilization free energies and decreased subunit interaction and unfolding free energies on the order of 1–2 kcal/mol compared with the native enzyme. Thus, the free energy differences between oxidized and native conformers support the contention that the conformational changes are small. However, this form of the protein is preferentially degraded by E. coli proteases (47–50). Oxidative modification of amino acids localized at or near metal binding sites results in decreased stabilization free energies of metal binding to the individual subunits and the subsequent loss of stabilization free energies of the quaternary structure. Oxidative modification of amino acid residues localized at the n2 metal binding site results in an alteration of the ATP binding site and increased access of the active site-linked central loop region to cleavage by trypsin. The conformationally linked free energies of stabilization and conformational movements of the central loop region attributed to interactions with the substrates, ATP and glutamate, are substantially attenuated in the oxidized conformer. As a result, the free energy differences between native and oxidized GS are actually magnified in the presence of the intrinsic substrates. Thus, substrate interactions with the oxidized protein might not protect this form of the enzyme from proteolysis by in vivo proteases.

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REFERENCES

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