Metal-dependent Self-assembly of Protein Tubes from Escherichia coli Glutamine Synthetase

Cu²⁺ EPR STUDIES OF THE LIGATION AND STOICHIOMETRY OF INTERMOLECULAR METAL BINDING SITES*

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Escherichia coli glutamine synthetase (GS) is a decameric assembly of identical subunits arranged as two back-to-back hexagonal rings. In the presence of divalent metal ions, the decamers “stack” along their six-fold axis of symmetry to yield elongated tubes. This self-assembly process provides a useful model for probing metal-dependent protein-protein interactions. However, no direct spectroscopic or structural data have confirmed the identity of the ligands to the shared metal ions in “stacked” GS. Here, 9-GHz Cu²⁺ EPR studies have been used to probe the ligand structure and stoichiometry of the metal binding sites. The wild type protein, with N-terminal sequence (His-4)-X₄-(Met-8)-X₅-(His-12), exhibits a classic Cu²⁺-nitrogen spectrum, with 

\[ g \approx 2.06 \text{, } g_i \approx 2.24 \text{, and } A \approx 19.3 \times 10^{-3} \text{ cm}^{-1}. \]

No supperhyperfine structure is observed. The H4C mutant affords a spectrum that is the combination of two spectra at all stages of saturation. One of the overlapping spectra is nearly identical to the spectrum of wild type, and is due to His ligation. The second spectrum observed yields 

\[ g \approx 2.28 \text{ and } A \approx 17.1 \times 10^{-3} \text{ cm}^{-1}. \]

The linewidth and tensor values of the second component have been assigned to Cu²⁺ occupancy that is very similar to the second set of spectral features observed for H4C, and which is assigned to Cu²⁺-S ligation. No Cu²⁺-His ligation is apparent until the Cu²⁺/N-terminal helices ratio is >1.0. At saturation, the 

\[ g \approx 2.00-2.06 \text{ region of the spectrum is essentially a mirror image of the spectrum obtained with H4C, and is due to overlapping Cu²⁺-N and Cu²⁺-S EPR spectra.} \]

The M8L and M8C mutants were also studied, in order to probe the role of position 8 in the N-terminal helix. Spectral parameters of these mutants are nearly identical to each other and to the wild type spectrum at saturating Cu²⁺, suggesting that Met-8 does not act as a direct metal ligand. Together, the results provide the first direct evidence for a binuclear metal ion site between each N-terminal helix pair at the GS-GS interface, with both His-4 and His-12 providing metal ligands.

Bacterial glutamine synthetases (GSs) are complex decameric aggregates arranged as two back-to-back hexameric rings of identical subunits (Fig. 1, A and B; Refs. 1 and 2). The resulting oligomers provide a circular complex that is similar to many other biologically assembled aggregates including GroEL, 20 S proteasomes, light harvesting complexes, and others (3). In addition, decameric GSs from some bacteria “stack” in the presence of divalent metal ions to form hollow tubes with a hydrophilic channel running through the self-assembled protein filaments (4–6). These protein tubes further assemble into three- and seven-stranded cables under some conditions, with specific lateral interactions between the disc-shaped decamers (6). Although the biological role for these processes has not been clearly defined, GS is a powerful model for design of metal-dependent protein docking (7, 8), and in principle, GS may represent a useful “scaffold” for fabrication of linear arrays of chromophores, redox components, or inorganic metal complexes, as suggested for other proteins, peptides, and DNA (9, 10).

The utility of GS, or any other protein, as a scaffold would be maximized if the self-assembly process could be controlled, and if biologically imposed structural constraints could be overcome. For example, GS variants have been engineered that afford protein tubes with “higher supramolecular order” than tubes obtained from wild type E. coli GS, as a result of engineered heterospecific interactions between electrostatically complementary mutants (11). In addition, we have explored in vitro methods for removing the biologically imposed symmetry of the GS dodecamer, which allow for control over the length of GS tubes (12). Also, the solution conditions that influence this metal-dependent protein-protein interaction have been previously characterized for wild type and site-directed mutants of E. coli GS, in order to understand factors that control this process (8, 13), and the thermodynamics of the reaction have been partially described (14). Together, these results have yielded GS variants with novel self-assembly properties and a useful structural model for the molecular mechanism of recognition between GS dodecamers.

Each subunit within the dodecamer contains an N-terminal helix, residues 1–14, with the helical axes approximately perpendicular to the six-fold symmetry axis that runs through the center of the ring structure (Fig. 1C). When two GS molecules are docked, helices are contributed from each dodecamer, forming six interdodecameric “helix-dimers.” Within the helices, His-4, Met-8, and His-12 provide potential ligands to metal ions

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1 The abbreviations used are: GS, glutamine synthetase; EPR, electron paramagnetic resonance; MSOX, L-methionine sulfoximine; PAGE, polyacrylamide gel electrophoresis; EM, electron microscopy.
that “bridge” these helix-dimers. On the basis of the previous characterization and model peptides with the sequence of the N-terminal helices (15), a model (Fig. 1D) that is consistent with the available data suggests that each of the six interdodecameric helix-dimer interactions provides a binuclear metal site, in which one metal ion will bind in a linear bidentate mode between the two His-4 side chains, and the second metal in a similar geometry between the two His-12 side chains. Thus, at each GS-GS interface, 12 metal ions are shared. In addition, the M-S8 side chains may provide ligands to either or both metals. This is based on the observation that the M8L mutant stacks poorly with all metal ions, whereas the M8C mutant stacks efficiently with some metals and poorly with others (13).

However, experimental approaches required to challenge this model have been inapplicable or unsuccessful. An inherent problem with site-directed mutagenesis, when not coupled to other experimental approaches, is the possibility that mutations that are remote from the amino acid substitution cause structural changes, which indirectly alter the function of interest. Therefore, the effects of mutation at His-4, Met-8, or His-12 provide only indirect evidence for their roles in the GS-GS stacking reaction. Optical spectroscopy of the metal centers and magnetic relaxation techniques relying on nuclei in the self-assembled GS tubes are hampered by the size and insolubility of these complexes. Thus, direct evidence for the nature of the protein ligands to the metals and the stoichiometry of the metal binding sites has not been elusive. In fact, no high resolution structural data for any GS dodecamer-dodecamer complex have been reported.

In contrast to optical techniques and protein-based nuclear relaxation methods, EPR provides a direct probe of metal environment that is not dependent on aqueous solubility or isotropic character of the supramolecular aggregate. Cu$^{2+}$ was previously demonstrated to be an efficient ion for directing the stacking reaction (8), but the spectroscopic properties of this metal bound at the GS-GS interface have not been exploited. Here, we employ Cu$^{2+}$ EPR as a probe of metal environment and binding stoichiometry of the intermolecular binding sites that mediate GS stacking. The results provide direct structural evidence in support of the binuclear nature of the interhelical sites and provide significant refinement of the previous model for dodecamer-dodecamer interactions of E. coli GS.

**MATERIALS AND METHODS**

**Mutagenesis and Protein Purification**—Site-directed mutagenesis and protein purification were as described previously (13), with the following modifications: ZnSO$_4$ precipitation, acetone precipitation, and (NH$_4$)$_2$SO$_4$/acid precipitation steps were omitted. Instead, after treatment with streptomycin sulfate, the cellular lysate was chromatographed over Blue Sepharose CL-6B (Amersham Pharmacia Biotech), and eluted with 40 ml of 20 mM ADP. The fractions were assayed by the γ-glutamyl transferase assay and those which showed greatest activity were pooled, concentrated, and poured over a G-25 gel-filtration column (Sigma). The eluent was assayed for activity by the γ-glutamyl transferase assay, and fractions that showed the greatest activity were pooled. Purity was assessed by SDS-PAGE to be >95%.

**MSOX Inactivation of GS**—Because GS is a metalloenzyme, the possibility exists that adventitious Cu$^{2+}$ could bind in the active site, displacing the ions which are normally found there, and interfering with the titration of the N-terminal binding sites. In order to minimize this possible interference, Mn$^{2+}$ ions were bound into the active site using the pseudo-irreversible inhibitor t-methionyl sulfonamide (MSOX), as described previously (5, 16, 17). Stocks of 100 mM MSOX and 100 mM Mg$^{2+}$-ATP (both acquired from Sigma) were prepared in deionized water. Purified GS was dialyzed against four 1-liter changes of buffer containing 50 mM HEPES (pH 7.2), 100 mM KCl, 20 mM MgCl$_2$, and 10 mM EDTA to remove Mn$^{2+}$ from the active site, replacing it with Mg$^{2+}$. This was followed by four 1-liter changes of buffer containing 50 mM HEPES (pH 7.2), 100 mM KCl, and 10 mM MgCl$_2$. Protein solution containing >10 mg of GS was concentrated to 4.5 ml total volume, after which 250 μl of MSOX and 250 μl of Mg$^{2+}$-ATP were added and the solution incubated for 1 h at 37 °C. After incubation, the protein solution was dialyzed against four 1-liter changes of buffer containing 50 mM HEPES (pH 7.2), 100 mM KCl, and 10 mM MgCl$_2$.

**Preparation of Samples for EPR Titrations**—The MSOX inactivated GS solution was concentrated to 5.25 mg/ml protein, or approximately 103 μM subunits (determined spectroscopically). Individual stocks of Cu$^{2+}$ (CH$_3$COO)$_2$, ranging from 0 to 5 mM Cu$^{2+}$ in 250 or 500 μM increments, were prepared from a central stock of 0.1 mM cupric acetate. Aliquots of 6 μl of each Cu$^{2+}$ stock were added to 144 μg of GS protein, bringing the total volume to 50 μl and the protein concentration to 5.1 mg/ml, or approximately 100 μM subunits. This resulted in a titration range from 0 to 200 μM Cu$^{2+}$, or 0–2.0 eq of Cu$^{2+}$ with respect to protein concentration. The samples were then placed in EPR sample tubes and stored in the refrigerator at 4 °C overnight.

**EPR Spectroscopy**—EPR spectroscopy was performed on a Bruker EMX X-band spectrometer. The following parameters were utilized for all experiments: the frequency was 9.41 GHz, the power was 63.31 mW, the modulation frequency was 100 kHz, the modulation amplitude was 10 Gauss, the time constant was 40.96 ms, and the conversion time was 163.84 ms. All spectra were recorded at 153 K, using liquid nitrogen as coolant.

**RESULTS**

We have suggested previously, based on turbidity measurements, that Cu$^{2+}$ induces GS dodecamers to self-assemble into extended tubes (8, 13). The self-assembly process was monitored by light scattering and transmission EM. Shown here are representative transmission EM micrographs of the wild type GS in the presence and absence of 20 μM Cu$^{2+}$ (Fig. 2). The EM micrographs clearly demonstrate the Cu$^{2+}$-dependent formation of GS tubes under conditions similar to those used for the EPR experiments. The EM experiments were performed at a 50-fold dilution with respect to GS concentration. The higher protein concentration is required for the EPR experiments, but this concentration affords undecipherable EM images due to side-to-side aggregation of tubes. We assume that these side-
to-side interactions do not interfere with the EPR signals characterized here. This assumption is supported by the data presented below.

100 μM wild type GS was titrated with 0–2.0 eq of Cu$^{2+}$, with respect to protein concentration, in incremental steps of 0.2 eq. This titration serves as a base line for the study of the various site-directed mutations to this metal binding site. The spectra of wild type GS (Fig. 3) is consistent with classically observed powder spectra of nitrogen-bound Cu$^{2+}$ ions. The $g_\parallel$, $A_\parallel$, and $g_\perp$ values observed are consistent with those observed for histidine- or imidazole-ligated Cu$^{2+}$ EPR standards (18–20). A small contribution from another species of Cu$^{2+}$ results in a perturbation of the spectrum, which is detectable at low equivalences of Cu$^{2+}$ but is obscured by the Cu$^{2+}$-protein signal as the Cu$^{2+}$ concentration increases. Comparison to a reference spectrum of 100 μM Cu$^{2+}$ in 50 mM HEPES (data not shown) indicated buffer-ligated Cu$^{2+}$ to be the most likely source of this minor interference.

Previously, it was suggested that Met-8 may play a role in the stabilization of these two sites (13). In an effort to elucidate the contribution from this residue, the M8C and M8L mutants were studied by EPR. In principle, the M8C mutation places a strong ligand in the vicinity of each of the proposed metal binding sites, and relieves some steric crowding. However, the much shorter cysteine side chain may not reach either Cu$^{2+}$ center. The M8L mutation removes the potential sulfur ligand from the vicinity of the two sites, and also adds steric bulk, which may perturb the geometry or solvation of either metal center. As mentioned previously, the M8C mutant was observed to stack more efficiently than wild type with some metals and less well with others, while the M8L mutant stacked less well than wild type regardless of the metal. In all cases, the M8C mutant was observed to stack with a larger apparent rate constant than the M8L mutant under similar reaction conditions. Interestingly, the $g_\parallel$, $A_\parallel$, and $g_\perp$ parameters observed for the two mutants were virtually identical, both to each other and to those observed for wild type GS (Table I). The most significant change observed was the marked difference in the lineshapes of the two mutants when compared with each other and to wild type. The small perturbation observed in the spectrum of wild type GS is greatly exaggerated in the M8L mutant (Fig. 4, top), and completely absent in the M8C mutant (Fig. 4, bottom). As a result of the variance in Cu$^{2+}$ buffer contribution, the linewidths of the spectra from the three proteins also show a trend, beginning with the narrow M8C spectra and widening as more contribution from buffer is observed in the wild type and M8L spectra.

In addition to studies in which we replaced individual metal ligands with other ligand types, we have also studied an H4A mutant (data not shown), in which the putative 4-site has been abolished. Similar to wild type, a spectral signal arising from buffer-bound Cu$^{2+}$ is observed at low concentrations of metal, but the spectrum of protein-bound copper is readily observed at higher concentrations. The Cu$^{2+}$ EPR spectra of the H4A mutant was nearly identical to those of the wild type enzyme, with the exception that the spectrum at 0.6 eq of Cu$^{2+}$/subunit of H4A GS is less intense than that observed for the wild type, indicating that less copper is bound. Presumably, this reflects cooperativity of binding the two metal ions when both His-4 and His-12 are present. For reasons which are not clear, an analogous mutant H12L, intended to completely remove the ligand at position 12, was expressed poorly. Therefore, we were unable to obtain sufficient quantities of this mutant to perform EPR. However, other substitutions at position 4 and 12 were characterized.

H4C and H12C mutants were studied in order to determine whether His-4 and His-12 are ligands to the Cu$^{2+}$ centers observed in the Cu$^{2+}$-GS spectrum, as proposed on the basis of previous work (8, 13). We hypothesized that the substitution of cysteine for histidine would afford a composite spectrum resulting from the ligation of Cu$^{2+}$ in two binding sites. Importantly, EPR spectral parameters for Cu$^{2+}$-S sites are not readily available from model compounds, due to the tendency of free thiols

![FIG. 2. Transmission electron micrographs of wild type GS in the presence and absence of Cu$^{2+}$:](Image 2)

![FIG. 3. 9-GHz EPR spectra of wild type GS in the presence of 0.6, 1.0, and 2.0 eq of Cu$^{2+}$/GS subunit.](Image 3)

### Table I

| Protein  | $g_\parallel$ | $g_\perp$ | $A_\parallel$ | Linewidth
|----------|---------------|------------|---------------|-----------
| Wild     | 2.06          | 2.24       | 19.3          | 123.2     |
| type     | 2.06          | 2.25       | 19.3          | 110.6     |
| M8C      | 2.06          | 2.24       | 19.1          | 138.8     |
| M8L      | 2.06          | 2.24       | 19.3          | 114.9     |
| H4A      | 2.06          | 2.24       | 19.3          | 135.9     |
| 4-site   | 2.06          | 2.28       | 17.1          | 79.2      |
| H12C     | 2.06          | 2.28       | 16.2          | 88.0      |
| 4-site   | 2.06          | 2.24       | 18.2          | 129.0     |
to reduce the Cu$^{2+}$ to Cu$^{+}$. Similarly, Cu$^{2+}$-S sites in proteins are much less well characterized than Cu$^{2+}$-N sites. Therefore, it is not straightforward to predict the spectral parameters to be expected for Cys-12 or Cys-4 ligation. In fact, a mutation-induced change in the spectrum would not only provide strong evidence for Cys ligation at the GS-GS interface, but may also provide a useful spectroscopic benchmark for Cys-ligated Cu$^{2+}$ sites in other proteins.

Titration of the H4C mutant resulted in a spectrum composed of two overlapping signals (Fig. 5, top). The double minimum in the $g_z$ region is indicative of two distinct sites, as is the weak second set of hyperfine lines. This composite of two distinct spectral centers is apparent throughout the titration of the H4C mutant. The H12C mutant, on the other hand, exhibited strikingly different behavior (Fig. 5, bottom). At concentrations of metal below 1.0 eq/helix, only one copper signal is observed, based on the presence of only one set of hyperfine lines and only one minimum in the $g_z$ spectral region. At approximately 1.0 eq/helix, however, the appearance of a second minimum in the $g_z$ region and a second set of hyperfine lines, accompanied by a shift in the $g_z$ value, indicates the filling of a second, spectrally distinct site. The appearance of a second set of spectral features upon substitution of one ligand type for another in the mutant proteins provides the first direct evidence for the identity of the ligands to Cu$^{2+}$ and to the stoichiometry of Cu$^{2+}$ binding.

**DISCUSSION**

Cu$^{2+}$ EPR spectroscopy has been used to determine the ligand identity and stoichiometry of the metal binding sites that are generated when N-terminal helices from GS subunits in adjacent dodecamers are juxtaposed in the stacked complex. Specifically, the results provide direct spectroscopic evidence that His-4 and His-12 each provide ligands to the metal ions bound at the dodecamer-dodecamer interface. Furthermore, the results show that, although Met-8 does not provide a direct metal ligand, it participates indirectly in the relative stabilization of the two sites.

The behavior of the M8L and M8C mutants indicates a role for Met-8 in the stabilization of the two metal binding sites. Through comparison of these mutants to the wild type protein, a general trend may be observed. A spectral feature in the wild type enzyme EPR spectrum (Fig. 3) is observed to be more prominent at low metal equivalency, but is masked at high equivalency of metal. The position of this spectral feature, centered at $g = 2.03$, corresponds exactly to the position of a prominent line in the spectrum of a Cu$^{2+}$-HEPES buffer control. That this interference from buffer-bound copper should be more evident at lower concentrations of metal is to be expected. This interference is also observed in the spectra from the titration of the M8L mutant (Fig. 4, top). However, in this case, the magnitude of the contribution from buffer is much more pronounced at all concentrations of copper than that found in the wild type spectrum at equal concentrations. In the case of the
M8C mutant, however (Fig. 4, bottom), the spectral feature around \( g = 2.03 \) is absent at all concentrations of \( \text{Cu}^{2+} \). Importantly, the \( g_i \), \( g_f \), and \( A_i \) values for the protein-derived ligands do not vary significantly as a result of these mutations. This suggests that no metal-ligand bond is formed between the side chain of the residue at position 8 and the \( \text{Cu}^{2+} \) at either site. Rather, the only effect of mutation at this site is a change in linewidth due to incorporation of varying amounts of \( \text{Cu}^{2+} \)-buffer signal, and we conclude that the effect of mutation at position 8 of the N-terminal helix is limited to an influence on the relative affinity of the two metal binding sites by indirect processes. Unfortunately, EPR is a poor method for measuring \( K_d \) values, so quantitative comparison of the binding affinities for these and other mutants will require other methods.

Previously published work utilized an H4A mutant protein, in which one of the two metal ligands has been eliminated (13). This mutants stacks with apparent lower affinity for metal ions than wild type. Here, the spectra from the \( \text{Cu}^{2+} \) EPR titration of H4A GS were essentially identical those obtained with the wild type enzyme at higher concentrations of metal, i.e. 1.0 eq/subunit or greater. At 0.6 eq of \( \text{Cu}^{2+} \)/subunit, however, the spectrum of H4A GS exhibits the same lineshape as wild type, but is much less intense.

Because each of the putative metal binding sites in the N-terminal helix utilize two histidine ligands, the spectra observed for the two sites overlap to such a degree as to be essentially identical. However, the substitution of cysteines for one or the other of the histidines at the two binding sites was expected to yield spectra containing features of two distinct binding sites.

The H4C mutant protein (Fig. 5, top) exhibits \( \text{Cu}^{2+} \)-binding behavior unlike the wild type enzyme, or any of the position 8 mutants. At low equivalency of metal (0.2–0.6 eq of \( \text{Cu}^{2+} \)) the hyperfine region of the spectrum contains approximately equal contributions from two populations of \( \text{Cu}^{2+} \)-centers. The \( g = 2.00 \) to \( g = 2.06 \) region of the spectrum contains a nearly symmetrical double minimum and a local maximum at \( g = 2.01 \). These results are most consistent with the simultaneous ligation of \( \text{Cu}^{2+} \) by the cysteines at positions 4 and the histidyl side chains at positions 12 of the N-terminal helices. At concentrations of \( \text{Cu}^{2+} \) larger than 0.6 eq, however, one set of hyperfine lines (those corresponding to a native-like \( \text{Cu}^{2+}\)-His site) becomes dominant. This causes an asymmetry in the \( g = 2.00–2.06 \) region of the spectrum, with the upfield minimum becoming more intense and a migration of the local maximum downfield to a value of \( g = 2.02 \). This behavior is due to a greater spectral contribution at higher concentrations of \( \text{Cu}^{2+} \) from the His site at positions 12 of the helix than that of the Cys site at positions 4.

The H12C mutant protein provides the most striking evidence that there are two independent metal binding sites in the N-terminal helix. Furthermore, unlike the case of the H4C mutant, the two sites appear to fill sequentially (Fig. 5, bottom). At concentrations below 1.0 eq of \( \text{Cu}^{2+} \), the spectrum of the copper-bound H12C protein appears to be an almost homogeneous spectrum with one set of hyperfine lines and one minimum in the \( g \) region. A minor perturbation due to a small amount of interference from \( \text{Cu}^{2+} \)-buffer complexes is observed at \( g = 2.03 \), as was previously observed in the wild type titration. This single set of spectral features most likely corresponds to the binding of a single population of \( \text{Cu}^{2+} \) to the cysteines engineered into the protein at positions 12 of the N-terminal helix. At approximately 1.0 eq of \( \text{Cu}^{2+} \), however, a second set of hyperfine lines begin to appear, accompanied by a second minimum. With increasing \( \text{Cu}^{2+} \), the second set of spectral features eventually results in a local maximum at approximately \( g = 2.02 \). The addition of a second population of bound copper also results in a very small but significant upfield migration of the \( g \) value. Whereas in the case of the H4C mutant, where both sites are populated to some extent at all concentrations of \( \text{Cu}^{2+} \) and as a result the averaged \( g \) value of the two sites does not significantly vary, the gradual addition of a second population of bound copper with a slightly different \( g \) value results in the observed change for the H12C mutant. These spectral changes correspond to the binding of a second, spectrally distinct, population of \( \text{Cu}^{2+} \) to the native histidines at positions 4 of the N-terminal helices. This second observed set of spectral features, however, do not attain the same magnitude as those of the features observed below 1.0 eq of \( \text{Cu}^{2+} \). This result was also observed in a titration with steps of 0.1 eq of \( \text{Cu}^{2+} \), and the results were identical; the appearance of the second set of spectral features begins at 1.0 eq of \( \text{Cu}^{2+} \), with the second set never reaching the same intensity as the first.

On the basis of these results, we conclude that the N-terminal helix of glutamine synthetase contains two distinct metal binding sites, at positions 4 and 12 of the helices. These two sites do not appear to interact electronically, as there is no evidence of anti-ferromagnetic exchange between them. This is not surprising in light of the distance between metal ions at these sites, ~13 Å, and the presence of residues between them at position 8 of the helix, which do not directly participate in metal binding. Furthermore, the results allow us to suggest that the 12-site is preferred over the position 4-site in the N-terminal metal binding region of GS. In both mutants where one of the native histidines was replaced with cysteine, spectral features from a \( \text{Cu}^{2+}\)-His site and a \( \text{Cu}^{2+}\)-Cys site are observed. In the case of the H4C mutant, both sites appear to be nearly equally populated at low equivalency of metal, but at intermediate to high concentrations, the spectral features arising from copper bound to the native histidines at the 12-site become dominant. In the case of the H12C mutant, the difference is more striking: The Cys site at position 12 fills completely before the His site at position 4 is occupied by \( \text{Cu}^{2+} \). In both cases, the spectral features derived from the copper-filled 12-site are more intense than those arising from the copper-filled 4-site. This observation leads us to suggest that the 12-site binds copper sufficiently tightly to yield full occupancy of the site. In contrast, binding of copper at the 4-site may be sufficiently lower in affinity to result in partial occupancy on the EPR time scale at the highest \( \text{Cu}^{2+} \) concentrations used. Notably, once an apparent maximum spectral intensity of the 4-site was achieved, the spectral contribution from that site did not increase with increasing concentration of metal ion. If partial occupancy is the cause for the decreased intensity, then it must be due to rapid exchange of the \( \text{Cu}^{2+}-\text{N} \) or \( \text{Cu}^{2+}-\text{S} \) at the 4-site with bulk solvated \( \text{Cu}^{2+} \), rather than failure to saturate the site. That is, binding of \( \text{Cu}^{2+} \) at the 4-site may be sufficiently dynamic to account for decreased intensity.

A potential explanation for this behavior is apparent from a model for the GS-GS complex based on the crystal structure of the \textit{S. typhimurium} protein (Fig. 1). The spectra observed for these sites by EPR are consistent with those of nitrogen bound copper ions (18–21). In this case, histidines bind \( \text{Cu}^{2+} \) in a linear bidentate arrangement, functioning as the dominant ligands, while contacts to bulk water are presumed to provide the rest of the ligand field, as there is no evidence of participation by other protein-derived ligands. Because of this geometry, the conformations adopted by the ligands at the 4 and 12 positions are critical to their ability to bind metal. According to our model, the histidines at position 12 are directed between the helices at an angle very close to optimal for this type of binding. Hence, \( \text{Cu}^{2+} \) can bind at the 12-site with a minimum
of induced strain. The histidines at position 4, however, are not so ideally positioned. Due to the helical twist, the side chains of position 4 are directed to either side of the plane defined by the two helical axes. As a result, alignment of these two histidines to adopt ligand field geometry similar to that found at position 12 incurs some strain, rendering the position 4 site a less desirable ligand field. This model is consistent with our observation that Cu$^{2+}$-binding at position 12 is preferred relative to position 4. Adding a further level of complexity to the model, however, is the presence of glutamic acid residues in the immediate vicinity of each of the two histidines. Residues Glu-3 and Glu-11 can adopt conformations suitable for the formation of a hydrogen bond between the carboxylate group of the glutamate and N61 of the histidine imidazole ring opposite the metal-binding Ne2. This type of "off-side" hydrogen bond has been implicated in the stabilization of metal-imidazole bonds (22–24). In the case of GS, the formation of an off-side hydrogen bond stabilizes the near-optimal metal binding conformation of the His-12 ligands; however, the formation of a similar interaction between Glu-3 and His-4 results in the stabilization of a less ideal ligand field, as mentioned above. Thus, for the His-4 site, an intrahelical hydrogen bond may compete with the formation of His-4 required to form an interhelical metal binding site, and reduce the apparent affinity for metal. Another potential distinction between the two sites that might contribute to the observed difference in binding behavior is the presence of a glutamate residue at position 13 which has no structural analogue at position 5. Although Glu-13 is not situated well for hydrogen bonding to His-12, the presence of an additional negatively charged residue in the vicinity of the 12-site may contribute significantly to the difference in the electrostatic attraction of the two sites toward metal cations. In addition to these other factors, this contrast in electrostatics may be sufficient to provide differential affinity for the two sites.

It is useful to keep in mind that the site under study is not necessarily occupied by Cu$^{2+}$ ions in the native state of the protein, nor has it any catalytic or redox activity associated with it. Rather, it is a unique intermolecular metal binding site which will bind copper, or a variety of other metals. Because of this difference, it would not be entirely unexpected that this site would not conform completely to any classically described, native copper site type. Nevertheless, a comparison of the findings to classically described sites is appropriate. As mentioned above, the two sites in the N-terminal helix of GS act independently of each other, and there is no indication of anti-ferromagnetic exchange between them; therefore, a type 3 site can be ruled out. Similarly, the absence of a strong blue color corresponding to a large absorption at ~600 nm or very narrow hyperfine splitting characteristic of the blue-copper proteins allows us to rule out the type 1 site. Because the $A_I$ values for these sites are all greater than 1.4 x 10$^{-4}$ cm$^{-1}$ and no absorbance at $\lambda = 600$ nm is observed, these copper centers are best classified as type 2 sites. Moreover, in light of the paucity of well characterized Cu$^{2+}$-S systems resulting from their tendency to undergo redox reactions, our results are of considerable interest as models for EPR parameters in proteins with Cu$^{2+}$-S bonds.

Most importantly, our results identify directly the metal-binding ligands that are responsible for the "stacking" phenomenon observed in E. coli glutamine synthetase. By replacing the native histidines that were previously reported as suspected ligands with other potential ligands, we have generated spectra that are composites of two distinct populations of bound copper. We have used these results to demonstrate that there are two separate binding sites for metal on the N-terminal helix, and that the site between positions 12 of a given "helix dimer" is preferred over the site formed between positions 4, based on the spectral features observed. We have also shown that the side chain at position 8 of the N-terminal helix does not directly ligate to either metal ion.

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