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Paramagnetic Probes of the Domain Structure of Histidine-Rich Glycoprotein†

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ABSTRACT: The interaction of Cu^{2+} and Fe^{3+}-mesoporphyrin with histidine-rich glycoprotein (HRG) from rabbit serum was examined spectroscopically. The first equivalent of Cu^{2+} binds to HRG producing a type II electron paramagnetic resonance (EPR) spectrum with $g_{\parallel} = 2.25$, $g_m = 2.05$, $A_{\parallel} = 0.019$ cm^{-1} (180 G), and superhyperfine along g_m. These spectral parameters suggest moderately covalent coordination of Cu^{2+} to the protein by nitrogens. With increasing Cu^{2+} the superhyperfine disappears; however, the g and A values change only marginally. The increase in EPR signal amplitude throughout the addition of 1-15 equiv of Cu^{2+} is linear and thereafter maximizes, suggesting 18-22 equiv are bound. In contrast, changes in the circular dichroism spectrum at 280 nm appear sigmoidal and can be interpreted as the binding of Cu^{2+} to two structurally distinct regions of the protein. Evidence for two structurally distinct binding domains is found by comparing EPR spectra of Cu^{2+} complexes of HRG with spectra from complexes of two of its major proteolysis products (peptides). After binding 1 equiv of Cu^{2+}, both the 30-kDa histidine-rich peptide and the native protein exhibit identical spectra including the pronounced superhyperfine. In contrast, the spectrum of the histidine-normal 45-kDa peptide with 1 equiv of Cu^{2+} bound lacks superhyperfine and parallels closely that of the native protein with 20 equiv bound. Finally, Fe^{3+}-mesoporphyrin binds to HRG exhibiting both high-spin ($g = 6.05$) and low-spin ($g_z = 2.94$, $g_y = 2.25$, $g_x = 1.50$) EPR resonances, and the latter imply bis(histidine) coordination. The amplitude of the low-spin g_z resonance decreases throughout the addition of 5-20 equiv of Cu^{2+}, and the Fe^{3+}-mesoporphyrin high-spin resonance increases and becomes rhombic at 10 equiv. The nitrogen superhyperfine is clearly visible even with Fe^{3+}-mesoporphyrin bound. Taken together, these data are consistent with Cu^{2+} binding to two structurally distinct metal-binding domains of HRG and a mostly sequential saturation of these domains. With increasing Cu^{2+} bound, Cu^{2+} coordination appears to progress from predominately histidine in the 30-kDa domain to aspartic acid and glutamic acid in the 45-kDa domain.

Although first isolated over 15 years ago (Heimburger et al., 1972), the physiological function(s) of the plasma glycoprotein HRG[1] is (are) yet to be determined. HRG interacts with plasminogen (Lijnen et al., 1980) and thrombospondin (Leung et al., 1984), with several divalent metals (Morgan, 1981, 1978), with hemes (Morgan, 1981; Tsutsui & Muller, 1982), and with organics such as rose bengal (Burch &

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[1] Abbreviations: HRG, histidine-rich glycoprotein; hemes, iron porphyrins; protoheme, iron protoporphyrin; mesoheme, iron mesoporphyrin; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; kDa, kilodalton(s); EPR, electron paramagnetic resonance; CD, circular dichroism.

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Morgan, 1985) and heparin (Kodie et al., 1982; Lijnen et al., 1983). Rabbit HRG is a 94-kDa single polypeptide chain and contains a large amount of histidine (11 mol %) and carbohydrate (Morgan, 1981; Tsutsui & Mueller, 1982). Both fluorescence quenching and UV spectroscopy suggest that 14–17 equiv[2] of Cu^{2+} bind to the protein with apparent K_d near 0.5 μM (Morgan, 1981). The binding of 25–30 equiv[2] of protoheme (K_d near 1 μM) is indicated by optical absorption spectroscopy at 400 nm; however, complete fluorescence quenching is induced by only 1–2 equiv. Furthermore, optical absorption studies suggest that the binding of protoheme can be inhibited partially by the interaction of HRG with metals including Cu^{2+} (Morgan, 1981). A recent report (Burch et al., 1987) that the interaction of HRG with heparin, a potent anticoagulant, is modulated by the presence of Cu^{2+} or Zn^{2+} is of potential physiological relevance. Indeed, HRG may be involved in a multitude of functions ranging from metal homeostasis to blood clotting, and metal binding may play a key role in some of this protein's functions.

Peptides of unusual amino acid composition have been isolated from rabbit HRG by plasmin cleavage (Morgan, 1985) and from human HRG by cyanogen bromide cleavage (Koide et al., 1985). Rabbit HRG yields a histidine-rich, 30-kDa peptide lacking aromatic amino acids and a 45-kDa histidine-normal peptide containing aromatics. By analogy with the amino acid sequence of human HRG (Koide et al., 1986), the 30-kDa peptide is identified as carboxy terminal and the 45-kDa peptide as amino terminal. Of particular relevance to this paper is the high Asx and Glx content of the 45-kDa peptide relative to the 30-kDa peptide from rabbit (Morgan, 1985). This result together with the sequence data from human HRG (Koide et al., 1986) implies a relatively high carboxyl content (Asp and Glu) for the 45-kDa peptide, but the majority of the histidine is found in the carboxy-terminal portion of the protein. Furthermore, spectroscopic studies of the 30-kDa peptide in comparison with the intact protein showed that the 30-kDa peptide represents one heme and metal-binding domain of HRG and have led to a description of the protein in terms of domains (Morgan, 1985). Koide et al. (1985) suggested an amino-terminal heparin binding domain, and Burch et al. (1987) showed the 30-kDa peptide was also implicated in heparin binding.

In order to further elucidate the interaction of ligands with HRG, the binding of the paramagnetic probes Cu^{2+} and mesoheme to the intact protein and its 30- and 45-kDa peptides was examined spectroscopically. These two peptides compose approximately 80% of the intact protein (Morgan, 1985); however, until now the 45-kDa peptide has not been examined extensively. The aims of this study were to determine the details of Cu^{2+} coordination to HRG and to examine in more detail the domain structure of this protein.

Materials and Methods

HRG was purified from rabbit serum by using ion-exchange chromatography (Morgan, 1978, 1981). Protein samples were lyophilized and stored at 4 °C. The concentration of protein was determined optically with an extinction coefficient of 55 mM^{-1} cm^{-1} at 277 nm (Heimburger et al., 1972) by using a mass of 94 kDa. The 30- and 45-kDa peptides from HRG were prepared by plasmin digestion as described by Morgan (1985). Cu^{2+} was added as $CuCl_2$ with concentration of the stock solution determined by direct titration with the primary standard Na_2EDTA and pyrocatechol violet indicator. Mesoheme (Porphyrin Products, Logan, UT) was dissolved in Me_2SO, and the concentration of the stock solution was determined by using 170 mM^{-1} cm^{-1} at 394 nm (Brown & Lantzke, 1969). After Cu^{2+} or mesoheme was mixed with protein, the samples were incubated for 2 min at room temperature, then frozen in dry ice/acetone, and stored in liquid nitrogen prior to recording EPR spectra. Afterward, the samples were thawed and the titration was continued.

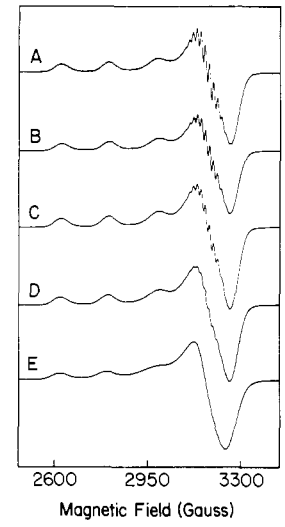

FIGURE 1: EPR spectra obtained after incubation of different concentrations of Cu^{2+} with HRG. The number of equivalents of Cu^{2+} available to complex with HRG was 1.0 (A), 2.9 (B), 4.9 (C), 9.7 (D), and 19.4 (E). HRG was dissolved in 0.05 M sodium phosphate, pH 7.4, and changed little in concentration (0.40–0.36 mM) during the Cu^{2+} titration. The settings of the spectrometer were as follows: microwave power, 20 mW; field modulation, 4 G; time constant, 0.3 s; and scan time, 8 min. The spectrometer gain was decreased from scans A through E so that the spectra remained approximately the same amplitude.

EPR spectra were recorded at 90 K on a Varian E-4 X-band spectrometer equipped with an E-257 variable-temperature (flow) apparatus. The effect of multiple freeze–thaw cycles on the EPR spectra was found to be negligible in the two cases examined. The g_m resonance was calculated from the magnetic field at which the EPR signal amplitude crossed the base line. Relative amounts of EPR-visible Cu^{2+} bound to HRG were quantitated by using the peak-to-trough amplitudes at g_m. Double integration of the most distinctly different EPR spectra obtained during the titration of HRG with Cu^{2+} and comparison with their peak-to-trough amplitudes verified that, to within 5%, the peak-to-trough amplitude at g_m was an accurate relative measure of the number of EPR-visible Cu^{2+} ions bound to HRG.

CD spectra were recorded with 1-cm cells at ambient temperature by using a Jasco J-500C spectropolarimeter equipped with a Model DP-500 data processor.

Results and Discussion

Interaction of Cu^{2+} with HRG. The EPR spectrum obtained after incubation of 1 equiv of Cu^{2+} with HRG in phosphate buffer is shown in Figure 1A. The spectrum is distinct from that given by buffered $CuCl_2$ alone and is described by the spectral parameters $g_{\parallel} = 2.25$, $g_m = 2.05$, and $A_{\parallel} = 0.019$ cm^{-1} (180 G). The differences demonstrate the formation of an HRG·Cu^{2+} complex as suggested by more indirect techniques (Morgan, 1981). The EPR parameters are similar to those measured for Cu^{2+} in laccase, galactose oxidase, carboxypeptidase, and other proteins (Malkin & Malmstrom, 1970). Since $A_{\parallel}$ is greater than 0.010 cm^{-1} in

[2] These numbers differ from those published previously by a factor of 94000/58000, which corrects for the most recent molecular weight determination for rabbit HRG (W. T. Morgan, unpublished results).

the HRG·Cu^{2+} complex, the metal is classified as type II. This EPR classification confirms optical absorption studies (Morgan, 1981) which show that the HRG complex is non-blue. Trends in $A_{\parallel}$ versus $g_{\parallel}$ values for many Cu^{2+} complexes of known coordination (Peisach & Blumberg, 1974) suggest that this Cu^{2+} is bound to HRG by either four nitrogens or a combination of nitrogens and oxygens, but not by four sulfurs or a combination of nitrogens and sulfurs. Furthermore, the spectral parameters of the HRG·Cu^{2+} complex are similar to those reported (Malmstrom & Vanngard, 1960) for models with Cu^{2+} bound by four histidines ($g_{\parallel}$ = 2.23, g_m = 2.06, and $A_{\parallel}$ = 0.018 cm^{-1}) or by four imidazoles ($g_{\parallel}$ = 2.27, g_m = 2.06, and $A_{\parallel}$ = 0.018 cm^{-1}) but are more distinct from those of four lysines ($g_{\parallel}$ = 2.21, g_m = 2.07, and $A_{\parallel}$ = 0.020 cm^{-1}) or mixed imidazole/peptide bond nitrogen coordination (Baffa et al., 1986; Bryce, 1966). The spectrum in Figure 1A also exhibits superhyperfine with approximately 10 lines averaging 14.8-G separation. This superhyperfine parallels closely that of Cu^{2+} bound to imidazole or pyridine with 14–15-G separation (Van Camp et al., 1981; Gersmann & Swalen, 1962) and is consistent with Cu^{2+} being bound to HRG by four equivalent nitrogens. By use of a molecular orbital formulation, the square of the $d_{x^2-y^2}$ orbital coefficient can be calculated from either the superhyperfine splitting or the spectral parameters $g_{\parallel}$, g_m, and $A_{\parallel}$ (Brill, 1977; Bryce, 1966), giving 0.77 and 0.81, respectively. These similar values emphasize the moderately covalent nature of the nitrogen–Cu^{2+} bonds. EPR spectra of HRG recorded before Cu^{2+} addition imply that only 0.03–0.09 equiv of Cu^{2+} copurify with the protein or arise as contaminants during preparation.

Figure 1 shows the changes in the EPR spectra with increasing Cu^{2+} and fixed HRG concentration. Aside from increasing amplitude, the most pronounced change is that the superhyperfine becomes increasingly obscured and after 10–15 equiv of Cu^{2+} are added is barely noticeable. However, there is little change in the overall shape of the spectra except that near 20 equiv added the $g_{\parallel}$ hyperfine appears to broaden slightly and $g_{\parallel}$ increases marginally to 2.26–2.27. These small changes are in contrast to the large spectral changes caused by strong dipolar and/or exchange interactions between protein-bound Cu^{2+} ions reported in, for example, aposuperoxide dismutase with Cu^{2+} addition (Fee & Briggs, 1975). This contrast suggests that dipolar and/or exchange interactions are small (or undetectable) with 20 or less equiv of Cu^{2+} bound to HRG. In addition, the ΔM_s = 2 transition noted in Cu^{2+}-substituted superoxide dismutase (Fee & Briggs, 1975) and nitric oxide treated ceruloplasmin (Van Leeuwen & Van Gelder, 1978) was not detected with HRG. Furthermore, throughout the addition of the first 20 equiv, distinct resonances that could be clearly attributed to a second kind of Cu^{2+} coordination, as was evident, for example, in the overlapping spectra of transferrin (Zweier, 1978), do not appear. This lack of resolution of distinctly different values of $g_{\parallel}$ and $A_{\parallel}$ can be explained by one of the following: (1) there are two or more EPR-detectable kinds of Cu^{2+} coordination with almost identical $g_{\parallel}$ and $A_{\parallel}$ values, some with and some without superhyperfine; (2) there is only one EPR-detectable kind of Cu^{2+} coordination, and dipolar broadening obscures the superhyperfine; or (3) there are two kinds of coordination present as described in (1) in addition to the effects of dipolar broadening. The two kinds of coordination might involve groups of different identity or identical groups bound in different conformations.

The variation in the amplitude of the g_m resonance (peak to trough) with increasing equivalents of Cu^{2+} added to HRG is given in Figure 2. The signal increases linearly throughout the addition of 15 equiv and then saturates in the range of 18–22 equiv. Dissociation constants were not determined from these data because there was virtually no free metal in solution at the high protein concentrations studied. This linear increase is in contrast to the erratic amplitude changes found with Cu^{2+} addition to *Neurospora* metallothionein (Beltramini et al., 1984) and argues against strong dipolar and/or exchange coupling in HRG. The binding of 18–22 Cu^{2+} equiv is verified by the CD titration data presented below. When taken together, these data suggest that 5 additional Cu^{2+} ions are bound over those revealed by previous UV and fluorescence spectral titrations (Morgan, 1981). Thus, results from different spectral techniques are consistent with two different kinds of Cu^{2+} coordination in HRG. The decrease in fluorescence intensity only reports the number of Cu^{2+} ions needed for quenching—not necessarily the total number bound. The changes in the UV spectrum likely depend upon metal–ligand charge-transfer transitions that would be specific to the identity and/or conformation of groups coordinating Cu^{2+} (Amundsen et al., 1977). Therefore, it is plausible that at least 5 Cu^{2+} ions are bound by groups not containing nitrogen. Alternatively, the total number of (near-neighbor) nitrogens bound by each Cu^{2+} may drop from 4 so that greater than 5 Cu^{2+} ions have mixed coordination. The shape of the titration curve in Figure 2 does not resolve a second kind of metal coordination in HRG. This does not preclude the existence of multiple kinds of coordination if the coordination becomes mixed or if the groups involved have similar dissociation constants as in the case of histidine– and carboxyl–Cu^{2+} complexes near neutral pH (Inczedy, 1976; Breslow, 1973).

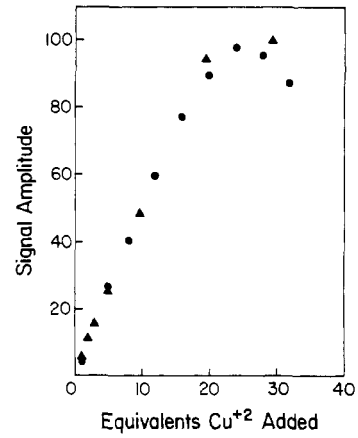

FIGURE 2: Signal intensity of the Cu^{2+} EPR spectrum with increasing Cu^{2+} added to HRG. The amplitude was measured from peak to trough at g_m and normalized to 50% at 10 equiv added. The concentration of HRG ranged from 0.22 to 0.19 mM (●) and from 0.40 to 0.36 mM (▲) during the Cu^{2+} titrations, and the plots were corrected for dilution.

The number of Cu^{2+} ions bound to HRG but not revealed through EPR spectroscopy is uncertain. Even after 25–30 equiv of Cu^{2+} are added to a solution containing HRG, there do not appear distinct resonances that differ from those previously discussed. The distinct but weak spectrum of Cu^{2+} in phosphate buffer [$g_{\parallel}$ = 2.39, g_m = 2.07, and $A_{\parallel}$ = 0.014 cm^{-1} (120 G)], similar to that reported for $Cu(H_2O)_6^{2+}$ (Falk et al., 1967), is absent. This absence suggests that, at greater than 20 equiv, Cu^{2+} coordinates to HRG, OH^-, and/or phosphate with no EPR signal. Both dipolar and/or exchange interactions could attenuate EPR signals (Smith & Pilbrow, 1974; Palmer, 1980). Precipitation was sometimes observed at greater than 25–30 equiv of Cu^{2+} added to HRG solutions in this study. Such precipitation may involve Cu^{2+}-induced

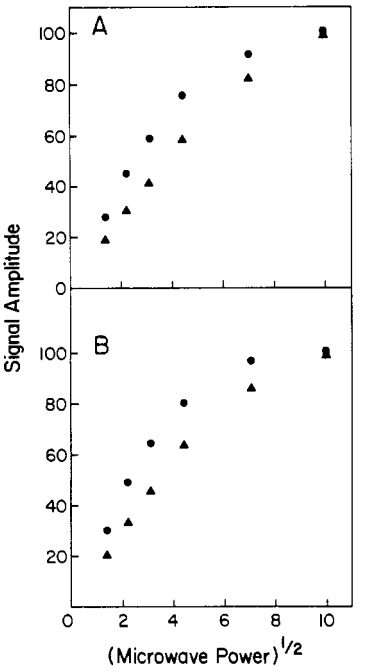

FIGURE 3: Microwave power saturation of EPR signals with 1 (●) and 20 (▲) equiv of Cu^{2+} bound per HRG. The amplitude was measured either from peak to trough (A) at g_m or from peak to base (B) at $g = 2.5$ and normalized to 100% at 100 mW.

aggregation of HRG through intermolecular bridging as suggested with ribonuclease (Breslow, 1973).

Figure 3 shows the dependence of the EPR signal amplitude of the HRG·Cu^{2+} complex on increasing microwave power for 1 and 20 equiv of Cu^{2+} bound. The signal is more easily saturable with 1 Cu^{2+} than with 20, suggesting that Cu^{2+} ions may be bound closely enough to enhance each other's relaxation through dipolar interaction. This interaction increases the power needed for saturation in the cardiac cytochrome oxidase (Ohnishi, 1982) and milk xanthene oxidase (Barber et al., 1982). Thus, it is plausible that attenuation of the superhyperfine in Figure 1 may be caused by dipolar broadening among (almost) identically coordinated Cu^{2+} ions bound to nitrogens. If the mechanism for disappearance of superhyperfine is dipolar broadening, the initial few Cu^{2+} ions are bound to HRG in relatively close proximity via nitrogen ligation, enhancing the notion of a histidine-rich binding domain (Morgan, 1981). The binding would likely occur randomly over several possible sites within this domain instead of in a well-defined sequence since there are no dipolar splittings indicative of fixed Cu^{2+} orientations (Palmer, 1980). However, it has been observed that dipole-induced spin-relaxation enhancement can occur even in the absence of dipolar broadening (Ohnishi et al., 1982). If this is the case with HRG, then the gradual disappearance of the superhyperfine is not inconsistent with progressive coordination of Cu^{2+} ions by oxygen after initial nitrogen coordination. It should be noted that absence of nitrogen superhyperfine is not conclusive evidence that nitrogen is not bound to Cu^{2+} (Froncisz & Hyde, 1980). Although nitrogens could remain bound and have a different conformation about the Cu^{2+}, it would be unexpected for nitrogen coordination to become free of superhyperfine without significant change in the other spectral parameters.

Oxygen coordination to some fraction of the Cu^{2+} ions by HRG is further supported by the following: (1) the similarity between $g_\parallel$ and $A_\parallel$ values of the HRG·Cu^{2+} complex and several model complexes with Cu^{2+} coordinated through oxygen listed in Table I; (2) the presence of several carboxyl groups (Asp, Glu) in HRG; (3) the discrepancy in the number

Table I: Spectral Parameters of Complexes of Cu^{2+} with HRG and with Models Having Ligands Coordinating through Oxygen

Cu^{2+} complex[a]	$g_\parallel$	$A_\parallel$ (cm^{-1})	g
HRG	2.25	0.019	2.05
bis(acetylacetonate)	2.27	0.016	2.05
saccharic acid	2.24	0.021	2.05
gluconic acid	2.27	0.019	2.06
tartrate	2.28	0.018	2.06

[a] Taken from this work, Maki and McGarvey (1958), Toy et al. (1971), and Boyd et al. (1973).

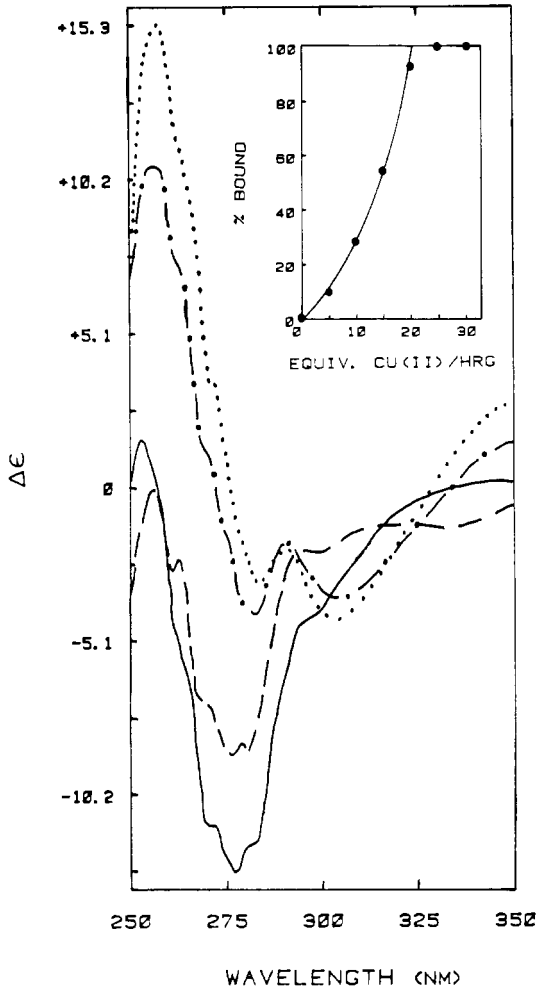

FIGURE 4: CD spectra in the near-UV of HRG as a function of added Cu^{2+}. The spectra are with no Cu^{2+} (—) and with 10 (– –), 20 (–·–), and 30 (···) equiv of Cu^{2+} added. The concentration of HRG was 23.9 μM in 0.05 M sodium phosphate, pH 7.4. The spectrometer scan speed was 5 nm/min, and the time constant was 2 s. The inset shows the change in ellipticity of HRG at 280 nm with increasing Cu^{2+}. The ordinate is expressed as percent of the largest magnitude deviation at 280 nm. This figure is a composite of three separate but similar titrations.

of Cu^{2+} ions bound to HRG mentioned above; (4) the similarity in K_d values for Cu^{2+} bound to histidine and carboxyl groups; and most significantly, (5) the distinct EPR spectra of Cu^{2+} bound to the 30- and 45-kDa peptides discussed below. Also of possible importance are model studies showing that $g_\parallel$ tends to increase as Cu^{2+} coordination changes from nitrogen to oxygen (Peisach & Blumberg, 1974). A marginal increase ($\Delta g_\parallel = 0.01$–$0.02$) apparently occurs in HRG. Neither the role of Cu^{2+} coordination by oxygen in HRG nor the role of these oxygens in interactions with other molecules has been addressed in the literature.

Whereas EPR is a site-specific probe of Cu^{2+} binding to HRG, CD provides information on overall protein confor-

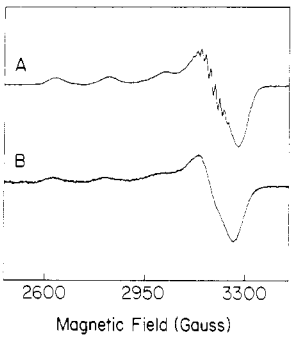

FIGURE 5: EPR spectra obtained after incubation of the 30- (A) and 45-kDa (B) peptides from HRG with 1 equiv of Cu^{2+}. The peptides were dissolved in sodium phosphate, pH 7.4, at concentrations of 0.40 and 0.17 mM, respectively. Instrument settings were as in Figure 1.

mation. The near-UV CD spectra of HRG exhibit a progressive change in ellipticity at 280 nm with increasing equivalents of Cu^{2+} added (Figure 4). This change suggests Cu^{2+}-induced alterations in the symmetry about the aromatic amino acids of the protein. A plot of the variation in ellipticity at 280 nm with increasing Cu^{2+} (inset, Figure 4) supports the binding of 20 equiv to HRG. However, in contrast to EPR, the CD titration is apparently sigmoidal with over 70% of the total change in ellipticity induced by the last 10 Cu^{2+} equiv. Although these data alone could be interpreted as cooperative Cu^{2+} binding, cooperativity is not evident in the EPR titration (Figure 2). An alternate interpretation is that Cu^{2+} binds to two structurally distinct regions of the protein. The first several equivalents of Cu^{2+} bind to the carboxy-terminal region which lacks aromatic amino acids (Morgan, 1985) and thus change the near-UV CD spectrum minimally. The latter equivalents of Cu^{2+} bind to the aromatic-rich, amino-terminal region (Morgan, 1985) and induce more pronounced CD changes. This sequential alteration of structurally distinct regions of HRG supports and extends previous work that suggests the protein contains structural domains (Morgan, 1981, 1985; Koide et al., 1985).

Interaction of Cu^{2+} with the Proteolysis Products of HRG. EPR spectroscopy of Cu^{2+} complexes of peptides isolated from HRG was used to further examine the idea of distinct metal-binding domains. The EPR spectrum of 1 equiv of Cu^{2+} bound to the 30-kDa histidine-rich peptide is given in Figure 5A. The spectrum is identical with that of 1 equiv bound to the intact protein including the pronounced superhyperfine. This spectral match implies that the 30-kDa peptide contains a Cu^{2+} binding site identical in composition and conformation to one located on HRG. Also, much of the structural integrity of the 30-kDa peptide is shown by its CD spectrum (Morgan, 1985) to be retained even after proteolysis and chromatographic separation, which supports the identification of this peptide as a structurally distinct metal-binding domain of the protein. Indeed, it is not uncommon for protein domains to retain their conformation and ligand binding characteristics after proteolysis. Furthermore, the coordination of Cu^{2+} by nitrogens in the 30-kDa peptide is consistent with its high histidine content (Morgan, 1985). In contrast, Figure 5B shows that 1 equiv of Cu^{2+} bound to the 45-kDa peptide lacks superhyperfine and appears most similar to HRG with 20 equiv of Cu^{2+} bound. Thus, dipolar broadening alone need not be associated with the absence of superhyperfine found in this study. Furthermore, a conformation change about the first Cu^{2+} bound that is induced by the binding of other Cu^{2+} ions need not be invoked to explain the disappearance of the nitrogen superhyperfine. The 45-kDa peptide contains several

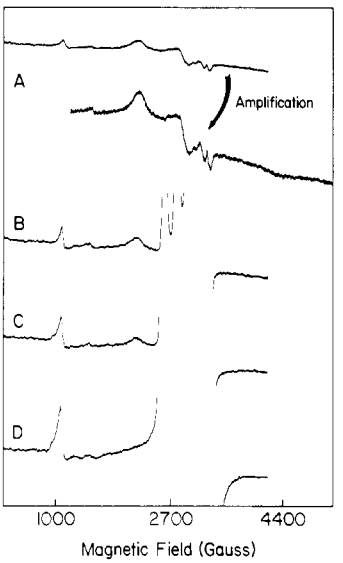

FIGURE 6: EPR spectra obtained after incubation of mesoheme or both mesoheme and Cu^{2+} with HRG. Mesoheme (3.5 equiv) was incubated with 0.20 mM HRG in (A). One equivalent of mesoheme was incubated with 0.63 mM HRG and then 2.0 (B), 9.9 (C), and 24.5 (D) equiv of Cu^{2+}. The buffer was 0.05 M sodium phosphate with 5–7% Me_2SO. The settings of the spectrometer were as follows: microwave power, 100 mW; field modulation, 10 G; time constant, 0.3 s; and scan time, 8 min. The spectrometer gain was unchanged in scans B–D.

carboxyl groups, and the superhyperfine-free spectrum is consistent with Cu^{2+} coordinated primarily to amino acids through oxygen.

The 45-kDa peptide apparently contains a Cu^{2+} binding site of similar composition and conformation to one located on native HRG and emphasizes a second structurally distinct metal-binding domain of the protein. Thus, the entire set of spectra in Figure 1, which describe the interaction of increasing Cu^{2+} with HRG, are apparently composed of varying contributions from the 30- and 45-kDa domains. This interpretation points to a model in which Cu^{2+} binds first to the histidine-rich, carboxy-terminal domain of HRG and at higher saturations through predominately oxygen coordination to the carboxyl-rich, amino-terminal domain. The ratio of histidine:carboxyl residues in rabbit HRG is estimated to be 6:1 and 1:5 in the 30- and 45-kDa domains (peptides), respectively (Morgan, 1985; Koide et al., 1986).

Interaction of Mesoheme and Cu^{2+} with HRG. The binding of mesoheme to HRG was used to probe further the interrelationships between the domains of this protein. The EPR spectrum that results from the incubation of 3 equiv of mesoheme with HRG is given in Figure 6A. Both high- and low-spin (mesoheme) species are present. Low-spin mesoheme is characterized by resonances at $g_z = 2.94$, $g_y = 2.25$, and $g_x = 1.50$, and a ligand field analysis (Blumberg & Peisach, 1971; Taylor, 1977; Muhoberac, 1984) of these g values ($\Delta/\lambda = 3.28$, $V/\Delta = 0.564$) implies that this fraction of mesoheme is bound to HRG by two uncharged histidines. The HRG–protoheme complex exhibits a low-spin optical absorption spectrum (Morgan, 1978), which can originate with a number of strong-field ligands. EPR spectroscopy, however, defines this ligation more precisely and emphasizes again the importance of histidine groups in this protein.

Addition of sufficient Cu^{2+} to the HRG–mesoheme complex alters both the high- and low-spin resonances, and these changes are outlined in Figure 6B–D for 1 equiv of mesoheme bound to the protein. The first 5 equiv of Cu^{2+} added cause little change in the shape or amplitude of the resonances at

g = 6.05 and g_z = 2.94. Similarly, the pronounced Cu^{2+} superhyperfine is observed with mesoheme bound (figure not given). At low Cu^{2+} concentrations, Cu^{2+} and mesoheme appear to interact with HRG at independent binding sites. Fluorescence quenching suggests that there is a preferred heme binding site on a 15-kDa peptide isolated from HRG and that the 15-kDa peptide is a subset of residues originally belonging to the amino-terminal 45-kDa domain (Burch & Morgan, 1985). Thus, EPR in combination with fluorescence studies suggests that, at low concentrations, mesoheme and Cu^{2+} bind preferentially to the 45- and 30-kDa domains, respectively, and interact minimally. However, addition of 5–20 equiv of Cu^{2+} causes complex spectral changes including a decrease in the g = 2.94 and an increase in the g = 6.05 resonance. At 10 equiv (Figure 6C) the high-spin resonance appears rhombic, and above 20 equiv (Figure 6D) the low-spin resonance is abolished. The added Cu^{2+} apparently causes some of the mesoheme on the 45-kDa domain to become coordinated by only one histidine. The second ligand is likely H_2O or possibly carboxyl, but the mechanism by which Cu^{2+} effects this change in ligation is unclear. The Cu^{2+} might coordinate a histidine directly and remove it from mesoheme coordination. Alternatively, changes in the CD spectra of HRG with Cu^{2+} are large, and a conformational change could remove a histidine from mesoheme without it becoming bound to Cu^{2+}. The rhombicity of the high-spin signal implies a directional inequivalence parallel to the heme plane which might arise from strain on axial ligands or heme in response to protein-imposed constraints (Blumberg & Peisach, 1971; Peisach et al., 1971). The change of one histidine from mesoheme to Cu^{2+} coordination does not preclude the majority of the Cu^{2+}–HRG interaction at higher equivalents of Cu^{2+} from involving mostly oxygen coordination. Still, the details of the simultaneous interaction of Cu^{2+} and mesoheme are complicated and are the object of current study.

In conclusion, 20 equiv of EPR-visible Cu^{2+} bind to HRG. Both EPR and CD studies of Cu^{2+} binding strongly suggest that HRG is characterized by two structurally distinct metal-binding domains. Previous workers labeled the 30-kDa domain histidine rich, and this study emphasizes the relatively carboxyl-rich composition of the 45-kDa domain. The binding of Cu^{2+} to HRG appears to progress from the 30-kDa domain with predominately histidine coordination to the 45-kDa domain with the involvement of carboxyl groups. Larger amounts of Cu^{2+} bound alter the bis(histidine) coordination of mesoheme causing a low- to high-spin transition. Together with the presence of Glu and Asp in the 45-kDa domain, both the disappearance of the nitrogen superhyperfine near 20 equiv of Cu^{2+} bound to HRG and the superhyperfine-free spectrum of 1 equiv of Cu^{2+} bound to the 45-kDa domain argue for increasing coordination of Cu^{2+} by carboxyl groups. Alternate explanations for elimination of the superhyperfine involve dipolar broadening and conformational changes, and some obscuration from these sources likely occurs. However, the evidence presented here does not rely on such sources to explain the bulk of the spectral changes. Characterization of the distribution of bound Cu^{2+} between nitrogen and carboxyl groups as well as between the 30- and 45-kDa domains may be important for the understanding of the interactions of HRG with its other ligands like heparin and plasminogen.

Registry No. Cu, 7440-50-8; L-Asp, 56-84-8; L-Glu, 56-86-0; mesoheme, 55297-44-4; L-histidine, 71-00-1.

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Heparin Cofactor II: cDNA Sequence, Chromosome Localization, Restriction Fragment Length Polymorphism, and Expression in *Escherichia coli*†

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ABSTRACT: Heparin cofactor II (HCII) is an inhibitor of thrombin in plasma that is activated by dermatan sulfate or heparin. An apparently full-length cDNA for HCII was isolated from a human liver λgt11 cDNA library. The cDNA consisted of 2215 base pairs (bp), including an open-reading frame of 1525 bp, a stop codon, a 3′-noncoding region of 654 bp, and a poly(A) tail. The deduced amino acid sequence contained a signal peptide of 19 amino acid residues and a mature protein of 480 amino acids. The sequence of HCII demonstrated homology with antithrombin III and other members of the α_1-antitrypsin superfamily. Blot hybridization of an HCII probe to DNA isolated from sorted human chromosomes indicated that the HCII gene is located on chromosome 22. Twenty human leukocyte DNA samples were digested with *Eco*RI, *Pst*I, *Hin*dIII, *Kpn*I, or *Bam*HI, and Southern blots of the digests were probed with HCII cDNA fragments. A restriction fragment length polymorphism was identified with *Bam*HI. A slightly truncated form of the cDNA, coding for Met-Ala instead of the N-terminal 18 amino acids of mature HCII, was cloned into the vector pKK233-2 and expressed in *Escherichia coli*. The resultant protein of apparent molecular weight 54 000 was identified on an immunoblot with ^{125}I-labeled anti-HCII antibodies. The recombinant HCII formed a complex with ^{125}I-thrombin in a reaction that required the presence of heparin or dermatan sulfate.

Heparin cofactor II (HCII)[1] is a 65 600-dalton glycoprotein in human plasma that inhibits the coagulation protease thrombin by forming an equimolar complex that is stable during SDS-PAGE (Tollefsen et al., 1982). HCII differs from other plasma protease inhibitors in its protease specificity. Unlike antithrombin III (ATIII), which inhibits all of the serine proteases of the intrinsic coagulation cascade, HCII specifically inhibits thrombin (Parker & Tollefsen, 1985). HCII also reacts slowly with the chymotrypsin-like protease cathepsin G (Parker & Tollefsen, 1985) and with chymotrypsin itself (Church et al., 1985).

HCII and ATIII differ from other protease inhibitors because their activities are stimulated approximately 1000-fold by certain glycosaminoglycans. Dermatan sulfate, heparin, and heparan sulfate increase thrombin inhibition by HCII in a dose-dependent manner, while chondroitin 4-sulfate, chondroitin 6-sulfate, keratan sulfate, and hyaluronic acid have no effect (Tollefsen et al., 1983). In contrast, only heparin and heparan sulfate increase the activity of ATIII. Thus, HCII is the principal inhibitor of thrombin in the presence of dermatan sulfate. Binding of heparin or dermatan sulfate to HCII appears to be required for the increased inhibitory activity observed in the presence of these glycosaminoglycans.

Recently, two partial cDNA clones encoding HCII have been identified. One consists of a 2.1-kb fragment which extends two nucleotides 5′ to the codon for the amino-terminal glycine of plasma HCII and contains 638 bp of 3′-noncoding sequence (Ragg, 1986). In our laboratory, a 1.2-kb cDNA was identified that contains 501 bp coding for the C-terminal 167 amino acids of HCII and 657 bp of 3′-noncoding sequence followed by a poly(A) tail (Inhorn & Tollefsen, 1986). These two sequences agreed entirely through the coding sequence. In this paper, we report a cDNA sequence that establishes the primary structure of the precursor of human HCII and have

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[1] Abbreviations: HCII, heparin cofactor II; ATIII, antithrombin III; SSC, 0.015 M sodium citrate/0.15 M sodium chloride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); kb, kilobase(s); EDTA, ethylenediaminetetraacetic acid.

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