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Interactions of the Histidine-Rich Glycoprotein of Serum with Metals†

William T. Morgan*

ABSTRACT: Histidine-rich glycoprotein (HRG) from serum binds copper (Cu^{2+}), zinc (Zn^{2+}), mercury (Hg^{2+}), cadmium (Cd^{2+}), nickel (Ni^{2+}), and cobalt (Co^{2+}). The protein from rabbit serum interacts with these divalent metals with high affinity, K_d near 1 μM, and an apparent stoichiometry of 10 metal ions bound per 58 000 molecular weight protein unit. Like HRG from human serum, rabbit HRG also binds heme with a K_d of about 1 μM. The interaction with metals inhibits the binding of heme and alters the fluorescence of the protein. In addition, Cu^{2+} affects the near-ultraviolet absorption and circular dichroic spectra of the protein. The apparent order of binding affinity is $Cu^{2+} \sim Hg^{2+} > Zn^{2+} \sim$ heme $\sim Ni^{2+} > Cd^{2+} > Co^{2+}$. No interaction between HRG and Mg^{2+}, Mn^{2+}, or Pb^{2+} was detected. The interactions of HRG with Zn^{2+} and Cd^{2+}, but not with other metals, show sigmoidal cooperative binding titrations. Moreover, only Zn^{2+} and Cd^{2+} cause an enhancement rather than a quenching of fluorescence. The interaction with Zn^{2+} is sensitive to pH: at pH 5.5 < 10% and at pH 6.0 only 50% of the binding measured at pH 7 remains. Histidine residues are implicated in the interaction with metals since the binding of Zn^{2+} is decreased in direct proportion to the ethoxyformylation of imidazole side chains by diethyl pyrocarbonate. HRG successfully competes for Zn^{2+} with metal-binding serum proteins like albumin and transferrin in equilibrium dialysis experiments. This together with the results above suggests that HRG may have a role in the transport or homeostasis of metals in vivo.

The transport in serum of essential nonferrous metals like copper and zinc is incompletely characterized. These metals play important roles in a wide variety of biological processes, and alterations in their metabolism have serious consequences (Prasad & Oberleas, 1976a). Also, metals increasingly contaminate the environment; those considered toxic, like cadmium and mercury, have deleterious effects not only on adults but also on fetuses and neonates (Prasad & Oberleas, 1976b). Several serum proteins, including albumin (Friedberg, 1975) and ceruloplasmin (Hsieh & Frieden, 1975), have been proposed to act as specific transport agents for essential and toxic metals. In addition, transferrin has been proposed to take part in the transport of both zinc and iron (Evans & Winter, 1975). However, although these proteins bind metals (Parisi & Vallee, 1970; Adham et al., 1977; McKee & Frieden, 1971), evidence that they deliver nonferrous metals to target tissues in vivo is lacking.

Histidine-rich glycoprotein (HRG)[1] from human serum was shown indirectly to interact with several nonferrous divalent metals (Morgan, 1978). Since imidazole is a powerful chelator of metals, HRG, which is nearly 10% by weight histidine (Heimburger et al., 1972), is an attractive candidate for a serum metal transport protein. An interesting property of human HRG is its decline during pregnancy to nearly one-half its usual concentration (0.125 mg/mL) in serum and its return to normal within a few days postpartum (Morgan et al., 1978). This decline and recovery are consistent with a role for HRG in providing metals to the fetus.

As one part of an investigation of the physicochemical and physiological properties of HRG, the ability of HRG isolated from rabbit serum to bind metals was examined directly by using a variety of techniques. This report presents evidence that rabbit HRG tightly binds certain nonferrous divalent metals in vitro and may therefore act in their transport in vivo.

Materials and Methods

Histidine-rich glycoprotein (HRG) was isolated from rabbit serum by procedures previously used for human HRG isolation (Morgan, 1978) which were based on those described by Heimburger et al. (1972). All buffers were treated with Chelex-100 (Bio-Rad Laboratories). In brief, the HRG was absorbed from serum onto CM-52 (Whatman) at pH 6 and eluted with 0.1 M sodium phosphate, pH 8. The HRG was then chromatographed on DE-52 (Whatman) at pH 7.4 with 15 mM sodium phosphate and a linear gradient to 0.5 M NaCl and rechromatographed on CM-52 at pH 6.5 with 50 mM sodium phosphate and a linear gradient to 0.5 M NaCl. HRG was detected in column fractions by using its ability to bind heme, thereby changing the absorbance of the ligand (Morgan, 1978), or by using specific antiserum raised in goats to rabbit HRG in a radial immunodiffusion assay (Morgan et al., 1978).

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[1] Abbreviations used: heme, iron protoporphyrin IX; HRG, histidine-rich glycoprotein; DEP, diethyl pyrocarbonate; HSA, human serum albumin; Cl_3CCOOH, trichloroacetic acid; Hepes, *N*-2-(hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid.

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The purity of HRG preparations (>95%) was assessed immunologically and by electrophoresis with and without sodium lauryl sulfate (Laemmli, 1970). HRG concentrations were determined spectrophotometrically by using an $E^{1\%}_{278}$ of 5.85 and a molecular weight by 58 000 (Heimburger et al., 1972). After isolation, HRG preparations were dialyzed against 100 mM sodium phosphate, pH 7.2. The HRG was then aliquoted into plastic tubes, frozen with liquid nitrogen, and stored at −20 °C.

Both rabbit and human HRG as isolated showed only negligible contamination with heavy metals (0.05 and 0.1 metal atom per protein molecule, respectively) when tested with dithizone reagent by using a method patterned after that of Song et al. (1976). This method was shown to provide reliable data with a sensitivity comparable to atomic absorption spectrophotometry with serum which contains proteins having tightly bound zinc and copper. Dithizone is not specific for zinc but will interact with several metals, including those of interest here. The major modificiation was the omission of KI to precipitate metals other than zinc in the procedure of Song et al. In brief, 5–50 mg of protein in 1 mL was precipitated with 0.15 mL of 100% trichloroacetic acid and the presence of released metals, e.g., Zn^{2+}, Cu^{2+}, Co^{2+}, or Ni^{2+}, assessed by spectral changes due to formation of the metal–dithizone chelate. Almost no metal was present. The sensitivity of the test was demonstrated by adding Zn^{2+} or Cu^{2+} to some HRG samples to a level of 1.0 metal atom per protein molecule before adding trichloroacetic acid. Some preparations of HRG were also tested for zinc and copper contamination with the same results as above by using a model 3010A anodic stripping voltameter from ESA Associates, Burlington, MA. Since the interaction between HRG and metal is pH sensitive with little binding of zinc occurring at pH 6 (see data below), the adsorption step at pH 6 could have removed any endogenous metal from the protein, and the purified, essentially metal-free, buffers used would preclude rebinding. No variations in the interaction with heme, Cu^{2+}, Zn^{2+}, or any other ligand were detected from preparation to preparation, indicating that no undetected metal contaminants were present. No heme was bound to isolated HRG as judged by the absence of absorption in the Soret region typical of the heme–HRG complex (see below).

Rabbit HRG, which was used to obtain the results presented here, is more readily available than human HRG since HRG is present in rabbit serum at levels (near 1 mg/mL) (W. T. Morgan, unpublished experiments) nearly 10 times those in human serum (0.125 mg/mL) (Morgan et al., 1978). No differences have been observed in the cases in which human HRG has been compared with rabbit HRG. Both proteins contain about 45 histidine residues per 58 000 molecular weight.

Metal solutions in 10 mM HCl were prepared gravimetrically from reagent-grade (Baker) metal acetate or chloride salts and stored in plastic tubes (Falcon 2059). Mercury solutions were prepared in 10 mM HNO_3. Certified standard solutions of metals were obtained from Fisher Scientific. Water was deionized and treated with a Milli-Q system (Millipore Corp.). All buffers were routinely treated with Chelex-100 (Bio-Rad Laboratories). The concentrations of heme (Eastman Organic Chemicals) solutions in dimethyl sulfoxide were assessed in the same solvent (Brown & Lantzke, 1969). ^{65}Zn (carrier free) was purchased from New England Nuclear and counted in a Searle Model 1185B well-type γ counter. Working solutions of ^{65}Zn were prepared by adding carrier-free ^{65}Zn to Zn stock solutions. The buffers used were 25 or 100 mM Hepes [*N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid], 0.25 M NaCl, pH 7.4, and 100 mM sodium phosphate, pH 7.2, treated with Chelex-100. No heavy-metal contamination in the buffer solutions was detected with the modified dithizone test described above (Song et al., 1976). However, Hepes buffer was not used in experiments in heme binding since this buffer inhibits heme binding by HRG apparently by interacting with the heme (W. T. Morgan, unpublished experiments).

Solutions of proteins other than HRG were prepared in the same buffers by weighing out dry, lyophilized material. Human serum albumin and iron-free transferrin were obtained from Behringwerke AG, West Germany. Human serum α_2-macroglobulin was kindly provided by the American Red Cross Blood Research Laboratory, Bethesda, MD, and rabbit hemopexin was prepared as previously described (Vretblad & Hjorth, 1977).

The interaction of HRG with metals and heme was assessed by several techniques. Absorption spectra were recorded by using a Cary 118C spectrophotometer at ambient temperature. Fluorescence spectra were recorded at 23 °C on a Perkin-Elmer MPF-44A spectrofluorometer by using the ratio mode. The excitation wavelength was 280 or 290 nm, and the emission maximum was at 338 nm for the rabbit protein (this work) and at 328 nm for human HRG (Morgan, 1978). For absorption, ligand added to equal volumes of buffer served as reference solutions; with fluorescence, ligand added to ovalbumin was used to correct for screening and inner-filter effects (Morgan et al., 1975), except for mercury which quenches ovalbumin fluorescence (Chen, 1976). Spectral measurements were usually made within minutes of mixing protein with ligand, and no changes after this time were noted. Changes in pH were negligible since only small volumes of metal solutions were added to buffered HRG solutions. The fractional saturation of HRG, α, is defined as the ratio of the observed ΔA or ΔF to the maximum ΔA or ΔF at a full saturation of the protein. The dissociation constant, K_d, was determined as $(1 - \alpha)[(L_0/\alpha) - P_0]$, where L_0 is the total ligand concentration and P_0 the total protein concentration, or by using a double-log (Chipman et al., 1967) or Scatchard (1949) method.

Circular dichroism spectra were obtained on a Cary 61 spectropolarimeter at 25 °C. After the spectrum of the apoprotein was recorded on 0.1 M sodium phosphate, pH 7.2, 10 equiv of Cu^{2+} or 15 equiv of Zn^{2+} was added, and the spectrum of the metal–HRG complex was recorded. The dynode voltage of the instrument was kept below 600 keV. Spectra in the near-UV were run by using a 1.0-cm path cell, and the data are reported as the molar ellipticity. In the far-UV, a 0.1-cm path cell was used, and the data are reported as the mean residue ellipticity by using a mean residue molecular weight of 115 calculated from the amino acid composition of rabbit HRG (W. T. Morgan, unpublished experiments).

Equilibrium dialysis was carried out in Plexiglas chambers of 0.2- or 2.0-mL total volume by using treated dialysis tubing as described (Morgan et al., 1975). ^{65}Zn was measured in aliquots taken from each side of the chamber; the bound ^{65}Zn was taken to be the difference between the ^{65}Zn in the presence of protein (bound + free) and ^{65}Zn in the other chamber (free). Recovery of ^{65}Zn exceeded 90% in experiments run for 16 h at room temperature. Serial measurements showed no changes after this time, indicating that equilibrium had been attained. In competition experiments, ^{65}Zn with an equivalent amount of HRG at 0.25 mg/mL (4.5 μM) was placed in one chamber

of an equilibrium dialysis apparatus and competing protein at 1–30 mg/mL in the other chamber. Hepes buffer (0.1 M, pH 7.4) with 0.25 M NaCl was used to minimize Donnan effects. In control experiments, the same amount of ^{65}Zn was placed with the individual proteins, and the ability of each protein to bind ^{65}Zn in the absence of competing protein was assessed. Samples taken after 16 h showed no changes in ^{65}Zn distribution, and the position of ^{65}Zn at the start of the experiment did not noticeably influence the results. All the proteins were tested for loosely bound heavy-metal content with a colorimetric assay (Song et al., 1976) with Zn^{2+} as the standard. After precipitation of the proteins with Cl_3CCOOH from the solutions used, less than 0.1 mol of metal per molecule was released from either HRG or rabbit hemopexin and less than 0.05 equiv from the other proteins.

Gel filtration experiments were conducted by using Bio-Gel P-10 or P-100 (Bio-Rad Laboratories) and 0.1 M sodium phosphate or 0.1 M Hepes buffer at flow rates of 15–30 mL/h. Bio-Gel did not bind nonspecifically with ^{65}Zn as found here with carbohydrate-based chromatographic media like Sephadex (Pharmacia). ^{65}Zn was measured in aliquots taken from collected fractions, and the absorbance at 280 nm was recorded by using an absorbance monitor with a flow cell. Metal–protein mixtures were incubated for 30 min prior to chromatography at room temperature.

Binding experiments with Bio-Gel P-10 (hereafter referred to as the gel exclusion binding assay) were performed at ambient temperature with a method based on that described by Hirose & Kano (1971). Solutions of protein alone and of metal alone were used to determine the exterior and interior volumes of the gel, respectively. After HRG was incubated with ^{65}Zn under the test conditions for 30 min, measured volumes of the solutions (usually 0.5 mL) were added to 100 mg of gel swollen in 1.0 mL of buffer in 12 × 75 mm polystyrene tubes. The tubes were gently mixed for 15 min on a gyratory mixer (Fisher Scientific) and then centrifuged for 1 min in a table-top centrifuge. The metal and protein contents of aliquots of the supernatant were measured. The recovery of HRG as A_{280} and the recovery of metal as ^{65}Zn were each greater than 90%. In addition, 1 mM EDTA completely abolished the interaction between ^{65}Zn and HRG but not between heme and HRG, showing that the assay accurately reflects full inhibition as well as full binding.

The gel exclusion technique was also used to study features of the HRG–metal interaction. HRG was preincubated with one metal for 30 min prior to incubation with a second metal for an equal time to assess competitive binding effects. In other experiments, the pH of the buffer was altered before ^{65}Zn was added. In others, HRG was treated with diethyl pyrocarbonate (Eastman Organic Chemical Co.), which has selective effects on histidine residues (Miles, 1977), before metal was added. The DEP to histidine ratios ranged up to 6:1, and little additional change in absorbance at 240 nm or effect on binding was observed at higher levels of DEP. In all cases, suitable controls were run to allow evaluation of the test samples. The gel exclusion technique was found to be general and rapid. For example, the fluorescence of proteins can be affected by changes in pH, and reaching equilibrium in dialysis requires several hours.

Results and Discussion

Interaction with Heme. The interaction of heme with rabbit HRG causes a quenching of tryptophan fluorescence and an alteration in the heme chromophore. The stoichiometries and apparent dissociation constants (K_d) are 1:1 and 0.5 μM with fluorescence (Figure 1, panel A) and near 15:1 and 1.1 μM with absorbance (Figure 1, panel B), respectively. These results were also obtained with human HRG (Morgan, 1978). The differences in stoichiometry determined from fluorescence and absorbance titrations may reflect the existence of a preferential site for heme binding or of a variety of binding sites at which heme undergoes energy transfers with the fluorophore(s). A preferential site would be expected to show a higher affinity for heme than the other sites, and the results here do not rule out such a site (Table I). The binding of 15 hemes compared to 10 metal atoms per protein molecule (see below) may indicate that some heme dimers interact with the protein or that the interaction with heme, requiring only 1 or 2 histidines per heme, is less restrictive than the interaction with metals, which can coordinate up to 4 histidines per metal (Sundberg & Martin, 1974). It must be borne in mind that two chemically distinct ligands which can bind to the same site on a protein may not have the same coordination with residues of the protein. The protein–ligand coordination may differ from heme to metal and from metal to metal. Although hemopexin, $K_d < 1$ pM (Hrkal et al., 1974), and albumin, $K_d < 10$ nM (Beaven et al., 1974), have a higher affinity for heme, heme can serve as a convenient chromophoric probe of the HRG molecule.

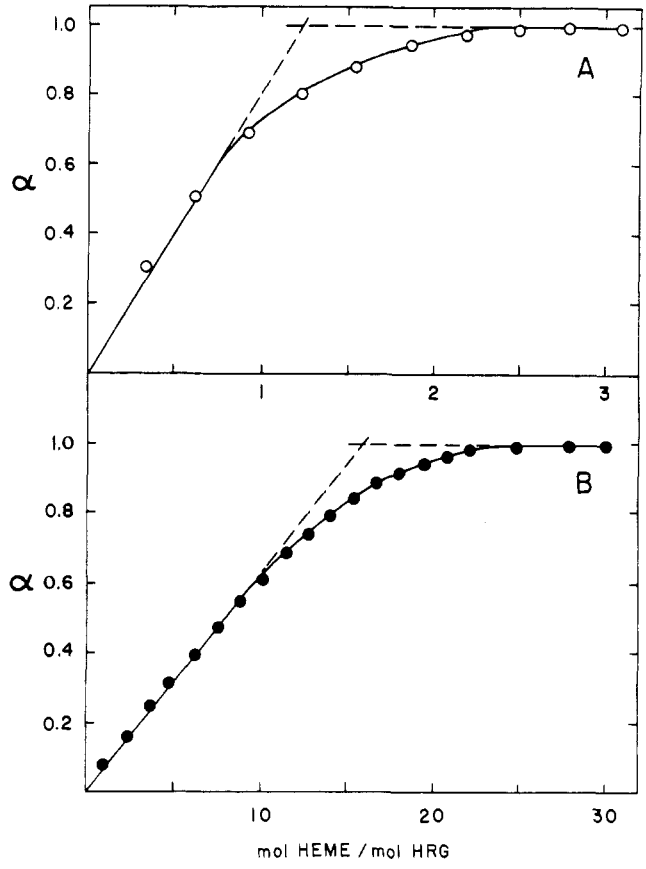

FIGURE 1: Binding of heme by rabbit HRG. (A) Quenching of fluorescence of tryptophan residues of HRG caused by increasing amounts of heme. (B) Change in absorbance of the heme chromophore produced by increasing amounts of heme. The fractional saturation, α, is defined as the ratio of the observed change in fluorescence or absorbance to the maximum change observed at full saturation. Note the change in units in the abscissa. The buffer was 0.1 M sodium phosphate, pH 7.15. The concentration of HRG was 2.9 μM in the fluorescence and 1.5 μM in the absorbance experiments.

Effect of Metals on Heme Binding. As shown in Figure 2, several divalent nonferrous metals effectively inhibit the formation of the heme–HRG complex as assessed by changes in the absorption of the heme chromophore in the Soret region λ_{max} near 414 nm. The isosbestic point observed near 387 nm

Table I: Interactions between Heme and Metals and Rabbit HRG

ligand	stoichiometry[a] (ligand/HRG)	K_d apparent[c] (μM)
heme	15[b]	1.1
	1.0	0.3
Cu^{2+}	9.5[b]	0.2
	10.5	0.2
Zn^{2+}	>10	ND[d]
Cd^{2+}	>10	ND[d]
Ni^{2+}	9.5	1.3
Hg^{2+}	10	0.4
Co^{2+}	10.5	2.1

[a] Stoichiometry determinations were made directly from titration data (Figures 1, 3, and 6) or from Scatchard-type (Scatchard, 1949) treatments. [b] These data were obtained from absorbance titrations by using the Soret band for heme and protein chromophores for copper. All the other data represent fluorescence titrations. [c] Apparent K_d values were determined from Scatchard (Scatchard, 1949) or double-log (Chipman et al., 1967) (Figure 7) treatment of the data. [d] Not determined. Due to the sigmoidal nature of the binding titrations of these ligands, the stoichiometry and K_d values must be viewed with caution. Rabbit HRG at 2.5 μM was fully saturated by about 20–25 equiv of these metals. The slope of the double-log plot of the data was 4.4 for Zn^{2+}, 2.8 for Cd^{2+}, and 1.0 ± 0.3 for all the others.

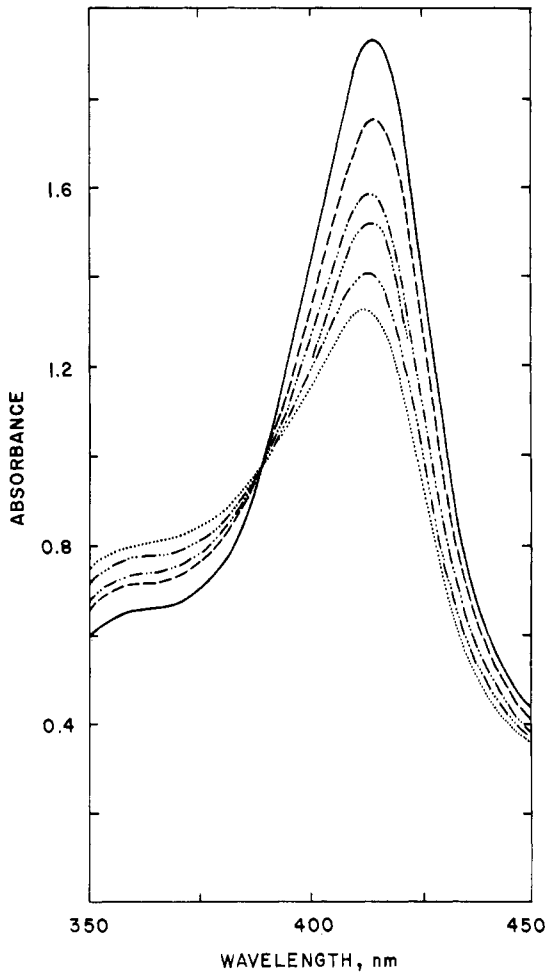

FIGURE 2: Binding of metals by rabbit HRG shown by competition with heme. The interaction between HRG (1.5 μM) in 0.1 M sodium phosphate buffer, pH 7.15, and divalent metals (22.5 μM) was demonstrated by adding heme (22.5 μM) 30 min after the metal and recording the absorption spectrum 4 h later. Shown are the following spectra: control, no metal (—); Cd^{2+} (- - -); Ni^{2+} (- · · · -); Cu^{2+} (- · - · - · -); Zn^{2+} (- · · - · · -); Hg^{2+} (· · ·). Co^{2+} (not shown) gave results very similar to those of Cd^{2+}.

indicates that only two species absorbing in this region are present, free and bound heme. The order of ability to inhibit

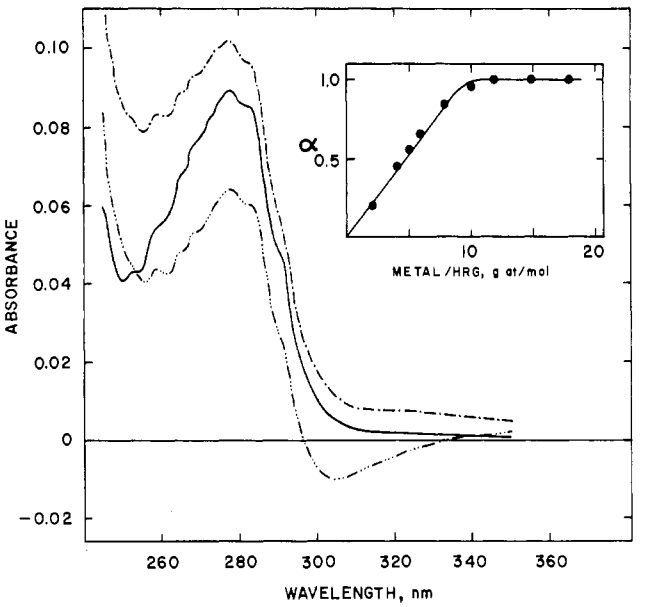

FIGURE 3: Absorption spectra of rabbit HRG and its copper complex. The spectra of HRG alone (—) and in the presence of 5 equiv of Cu^{2+} with either buffer (- - - -) or 5 equiv of Cu^{2+} (- · · · -) in the reference cuvette are depicted. Cu^{2+} alone under these conditions has a broad absorption band of low intensity with a maximum near 260 nm (not shown). The concentration of HRG was 3.05 μM, and the buffer was 0.1 M Hepes, pH 7.3. The inset shows the fractional saturation (α) of HRG with increasing amounts of Cu^{2+} observed in a difference spectra titration experiment under the same conditions. The decrease in absorbance at 277 nm was used to determine α.

heme binding is $Hg^{2+} \sim Cu^{2+} > Zn^{2+} > Ni^{2+} > Cd^{2+} \sim Co^{2+}$; no inhibition was seen with Mg^{2+}, Mn^{2+}, or Pb^{2+}. Similar results were obtained when metals were added to HRG after heme rather than before.

In titration experiments (not shown), a direct relationship was found between the amount of copper or zinc added to HRG and the decrease in bound heme, indicating competition for common binding sites. A 50% inhibition of binding of 15 equiv of heme was seen with 15 equiv of Cu^{2+} and 20 equiv of Zn^{2+} per mol of HRG. With the gel exclusion binding assay, the binding of ^{65}Zn was inhibited by heme, further supporting competition between metals and heme for at least some of the same sites on HRG.

Copper–HRG Absorption, Fluorescence, and Circular Dichroism. The interaction of copper (Cu^{2+}) with HRG was demonstrated directly by changes in absorption (Figure 3). The ultraviolet absorption and difference absorption spectra of HRG in the presence of 5 equiv of Cu^{2+} show perturbations at wavelengths below 285 nm. This is consistent with the interaction altering absorption bands of copper and/or the environment of tyrosine and phenylalanine residues. Since 15 equiv of Zn^{2+} had no effect on the absorption of HRG, the former explanation is preferred. The changes in absorbance produced by copper showed saturation (inset, Figure 3), with a binding stoichiometry near 10 Cu^{2+} per HRG and an apparent K_d near 0.5 μM. No absorption bands of copper–HRG were detected in the visible region, indicating that copper–HRG is a nonblue copper protein. This is not unexpected since blue (type I) copper proteins involve sulfur–copper coordination (Amundsen et al., 1977), and HRG has few cysteine residues (Morgan, 1978).

Copper also quenches the fluorescence of HRG as shown in Figure 4. The decrease is proportional to the amount of Cu^{2+} added, up to a saturating level near 10 equiv of copper (Figure 6), in agreement with the absorbance titration (Figure 3, inset). The fluorescence of rabbit HRG shows an excitation

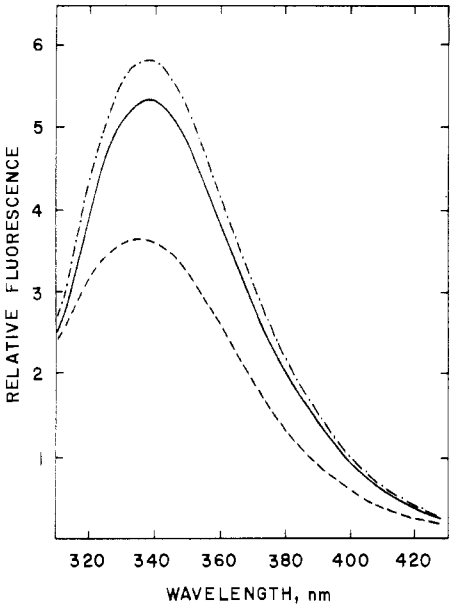

FIGURE 4: Fluorescence spectra of rabbit HRG and its copper and zinc complexes. The spectra of 2.9 μM HRG alone (—) and in the presence of 10 equiv of Cu^{2+} (---) or 15 equiv of Zn^{2+} (·····) are shown. Excitation was at 290 nm in 0.1 M Hepes buffer, pH 7.3. The Cu–HRG spectrum has been corrected for Cu^{2+} screening effects.

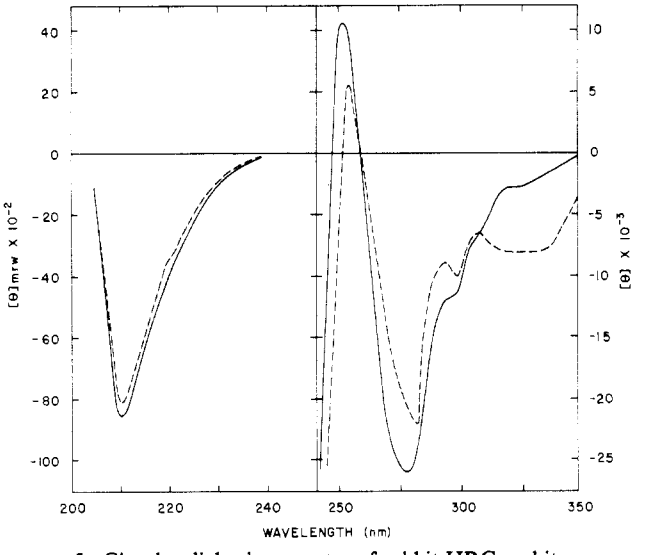

FIGURE 5: Circular dichroism spectra of rabbit HRG and its copper and zinc complexes. Spectra of rabbit HRG (19.5 μM) in 0.1 M sodium phosphate, pH 7.2, were recorded before (—) and after (---) addition of 10 equiv of Cu^{2+}. Spectra were also recorded before and after addition of 15 equiv of Zn^{2+} (not shown). The data are presented as molar ellipticity (deg·cm²·dmol⁻¹) in the near-ultraviolet (350–240 nm) and as mean residue ellipticity in the far-ultraviolet (240–200 nm).

maximum at 285 nm with a shoulder at 292 nm and an emission maximum at 338 nm (Figure 4), consistent with tryptophan being the predominant fluorophore. Human HRG has a similar excitation spectrum but an emission maximum at 328 nm (Morgan, 1978).

The interaction with copper also produces changes in the circular dichroic spectrum of rabbit HRG. The spectra of the apoprotein in the near- and far-ultraviolet regions (Figure 5) closely resemble those of human HRG (Morgan, 1978) with minima in ellipticity near 280 and 210 nm and a maximum near 250 nm. The interaction with Cu^{2+} produced distinct effects in the near-ultraviolet spectrum but only minor alterations in the far-ultraviolet spectrum (Figure 5). The near-ultraviolet changes may be due to optical activity of the

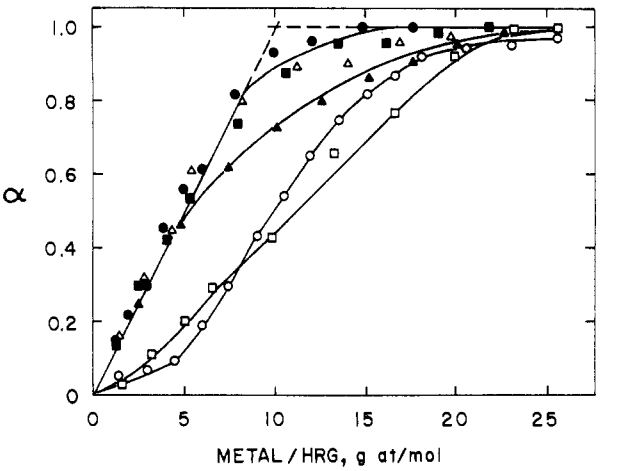

FIGURE 6: Titrations of metal–HRG interactions. Rabbit HRG (2.9 μM) in 0.1 M Hepes, pH 7.4, was titrated with Cu^{2+} (●), Zn^{2+} (○), Cd^{2+} (□), Hg^{2+} (■), Co^{2+} (▲), and Ni^{2+} (△) by using changes in fluorescence to monitor the interaction. Mg^{2+}, Mn^{2+}, and Pb^{2+} under the same conditions had no effect. See text for additional details.

absorption bands of copper in this spectral region specific to the copper–HRG complex (Figure 3). Alternatively, or in addition, the CD spectrum may arise from perturbation of the environment of a protein chromophore in the copper complex, reflecting a shift in the tertiary conformation. The small far-ultraviolet effect suggests that at most a minor change in the secondary conformation of the protein occurs even when up to 10 equiv of copper are bound. There is no detectable effect on the CD spectrum of HRG in either region when 15 equiv of Zn^{2+} is added to the protein (not shown), further supporting the existence of optically active absorption bands of copper.

Additional Effects of Metals on Fluorescence. Other divalent metals also alter the fluorescence of HRG, thereby allowing interactions to be assessed. These changes may reflect alterations in the environment of the fluorophores and energy transfers with metals which have charge-transfer absorption bands in this spectral region. Hg^{2+}, Ni^{2+}, and Co^{2+} quenched the fluorescence of HRG, although to a smaller extent than Cu^{2+}. The apparent stoichiometry of binding was near 10 metal ions per HRG molecule for these metals (Figure 6). Mg^{2+}, Mn^{2+}, and Pb^{2+}, which do not inhibit heme binding, also do not affect the emission of HRG.

The interactions between HRG and Zn^{2+} or Cd^{2+} also affect the fluorescence of the protein; however, an enhancement rather than a quenching of the tryptophan emission is found (Figure 4). This may be caused by the lack of charge-transfer bands of these metals. In addition, titration of the interaction of HRG with Zn^{2+} or Cd^{2+} showed a sigmoidal relationship between fluorescence and amount of Zn^{2+} present (Figure 6). In contrast, Hg^{2+}, the other member of periodic table group 2B, binds tightly to HRG but shows neither sigmoidy nor enhancement. This may be explained by the different properties of Zn and Cd compared with those of Hg (Basolo & Pearson, 1967; Cotton & Wilkinson, 1976). Scatchard plots of the Cd^{2+}- and Zn^{2+}–HRG fluorescence data as well as equilibrium dialysis data with ^{65}Zn (not shown) contain maxima and pass through the origin at $[metal]_{free} \rightarrow 0$, which rules out the presence of independent, noninteracting sites. The slope of a double-log plot (Figure 7) is 4.4 for Zn^{2+} and 2.8 for Cd^{2+}, indicating that these two metals are bound in a cooperative manner with interactions among the various sites. The stoichiometry of binding and apparent K_d values can be obtained only by extrapolation at high saturation levels and must be viewed with caution. Qualitatively, as judged from

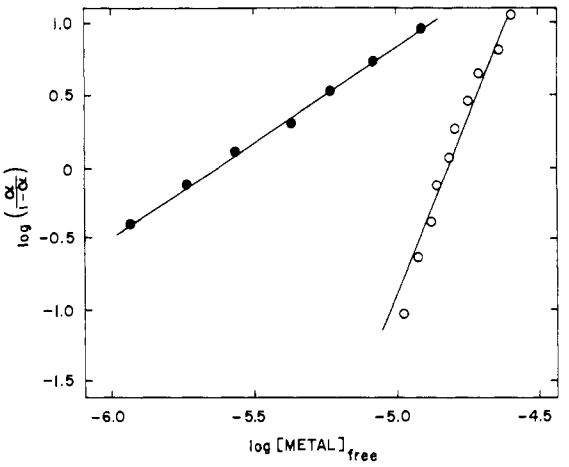

FIGURE 7: Double-log plots of the interaction between HRG and copper and zinc. The fluorescence of rabbit HRG was titrated with Cu^{2+} (●) and Zn^{2+} (○) under the conditions described in Figure 6.

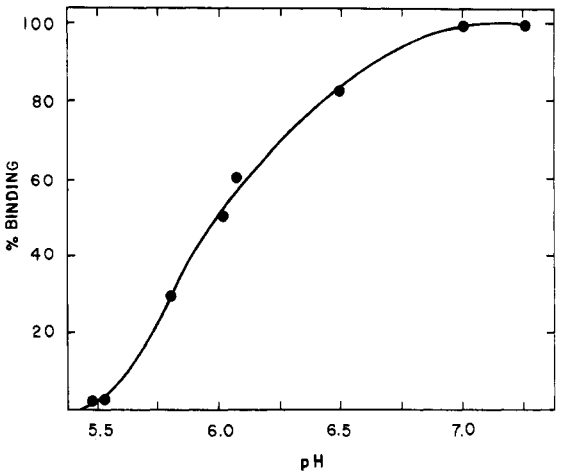

FIGURE 8: Effect of pH on the interaction of HRG with ^{65}Zn. Rabbit HRG (4.8 μM) in 0.1 M sodium phosphate buffer at the indicated pH was mixed with 20 equiv of ^{65}Zn and the extent of binding assessed by gel exclusion. Binding at pH 7.3 wad defined as 100%, and a buffer blank with no HRG present was taken as 0% binding.

the results of competition experiments (Figure 2), HRG seems to have an affinity of the same order for Cd^{2+} and Zn^{2+} as for heme, i.e., K_d near 1 μM. This cooperative binding of metal resembles the sigmoidal interaction of the multisubunit enzyme citrate lyase with Mn^{2+} (Sivaraman & Sivaraman, 1979). An apparent stoichiometry near 18:1 and a K_d of 45 μM were extrapolated from a Scatchard plot of the data. The Hill plot of the Mn^{2+}–lyase data showed a slope of 2.25, which was interpreted as showing significant positive cooperativity of binding.

The dissociation constants (K_d) of the ligand–HRG complexes and the stoichiometries of binding are summarized in Table I. In general, the apparent K_d values lie at or below 1 μM. Since the binding stoichiometries of the ligands are near 10:1, the effective binding concentration of HRG in serum is correspondingly increased 10-fold, and, thus, its potential to act as a physiologically significant metal binder is enhanced. The levels of HRG in human serum are about 0.15 mg/mL (2.5 μM) (Morgan et al., 1978) and about 1 mg/mL (15 μM) (W. T. Morgan, unpublished experiments) in rabbit serum. The cooperative binding of Zn^{2+} and Cd^{2+} may have physiological implications: at low levels of Zn^{2+} or Cd^{2+} in serum, perhaps less than 0.1 ppm or 1.5 μM, HRG would have little effect, but at higher levels, HRG might significantly affect the transport or homeostasis of these metals.

Competition between Metals. For determination of whether metals have binding sites in common on the HRG molecule, several types of competition experiments were performed. Addition of Cu^{2+} to the saturated Zn–HRG complex produced normal quenching of fluorescence, but addition of Zn^{2+} to the Cu–HRG complex had no effect, confirming that copper is bound with higher affinity than zinc. The copper–HRG complex is under investigation currently. The interaction between HRG and Zn^{2+} was chosen for further study here because zinc is a physiologically important metal, the interaction is not so tight as to prevent exchange, and the ^{65}Zn isotope has a usefully long half-life, unlike the short-lived isotopes of copper. In the gel exclusion assay, when 20 equiv of Zn^{2+} is added to HRG preincubated with 20 equiv of competing ligand, only Cu^{2+}, heme, Zn^{2+} itself, and Ni^{2+} caused a significant decrease in the amount of ^{65}Zn bound.

These results agree in general with predicted affinities based on the heme inhibition assay technique (Figure 2) and on the data in Table I. The metals tested were effective in inhibiting the HRG–heme interaction (Figure 2) with the order Cu ~ Hg > Zn ~ Ni > Cd ~ Co. The order of affinity on the basis of the apparent K_d values (Table I) is Cu ~ Hg > Ni > Co. The sigmoidal binding relationship, which makes K_d values for Cd^{2+} and Zn^{2+} difficult to evaluate, precludes ranking them by this criterion. However, Cd^{2+} appears to be bound more loosely than Zn^{2+} by HRG. For example, Cd^{2+} does not inhibit heme binding as well as Zn^{2+} does, and Cd^{2+} does not extensively inhibit the binding of ^{65}Zn in the gel exclusion assay. The reason for the weak inhibition of ^{65}Zn binding by Hg^{2+} is not clear, although Hg^{2+} appears to interact strongly with the gel.

Although heme and metals appear to bind at the same sites, there are apparent differences among these sites and interactions. Some of this difference can be attributed to the existence of different subsets of binding sites. That is, there is a set of 15 sites for heme and 10 sites for metals. These two overlap but are not identical. The question remains as to the extent of overlap. Can metals entirely abolish heme binding? It does not appear so, thus some sites for heme only may exist as implied by the higher binding ratio. The converse may not be true. In addition, differences in coordination between ligand and protein (see above), and possible interactions between binding sites, complicate the picture even more. Additional work is in progress addressing these questions, but certain tentative conclusions are supported. All the metal ions probably share the same set of binding sites although some nonoverlapping sites may exist. This possibility is easier to reconcile than each metal having its own set of sites. However, since each metal may involve a different group of protein ligands (histidine residues) in a different way, the details of the interaction of each metal with the protein will vary, even to the extent of showing sigmoidal binding only in certain cases.

Effects of pH and Diethyl Pyrocarbonate. The pH dependence of the interaction of HRG with ^{65}Zn is depicted in Figure 8. A sharp decrease in binding is seen below pH 7 with a midpoint near pH 6.0 and no binding at pH 5.5. This decrease is consistent with titration of histidine residues in this pH region, but may also reflect changes in protein conformation. Attempts to examine the effects of alkaline pH on the interaction were unsuccessful due to the insolubility of these metals under alkaline conditions.

The interaction of HRG with ^{65}Zn is inhibited by treatment of the protein with diethyl pyrocarbonate (DEP), a reagent which can be used to selectively modify histidine residues

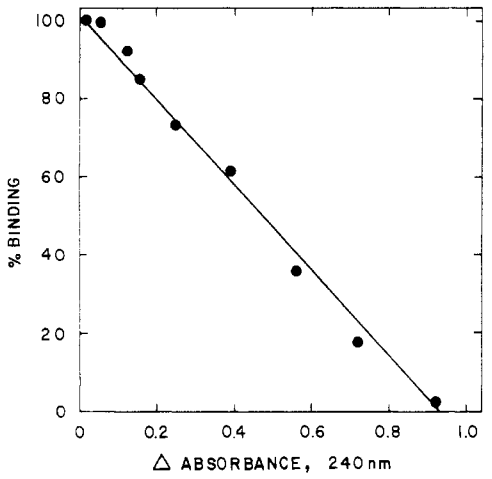

FIGURE 9: Effect of diethyl pyrocarbonate on the interaction of HRG with ^{65}Zn. Portions of rabbit HRG (4.8 μM) in 0.1 M sodium phosphate buffer, pH 7.0, were treated with various amounts of DEP for 60 min at room temperature. Binding of ^{65}Zn (20 equiv added) was assessed in one part of each reaction mixture by gel exclusion and the extent of carbethoxylation of histidine monitored in another portion from the change in absorbance at 240 nm (Miles, 1977). Binding in the absence of treatment was defined as 100%, and a buffer blank with no HRG present was taken as 0% binding.

(Miles, 1977). The decrease in interaction with ^{65}Zn was directly proportional to the extent of histidine modification (Figure 9), as judged by the increased absorbance at 240 nm (Miles, 1977). As many as 35 of the 45–50 histidine residues were modified. This strongly supports the participation of histidine residues of HRG in the metal-binding action of the protein, especially since no change in the near- or far-ultraviolet circular dichroism spectra of HRG was found even at high levels of modification (data not shown). A more detailed examination of the effects of pH on the conformation of HRG and of other histidine-selective modification agents is in progress.

Comparison with Other Serum Proteins. The zinc-binding ability of HRG was compared directly with that of other serum proteins. In preliminary experiments, ^{65}Zn co-chromatographed with HRG on Bio-Gel P-100 gel filtration media but not with human serum albumin, suggesting that HRG has a higher affinity for Zn^{2+} than does HSA. All the proteins examined bound Zn^{2+} in equilibrium dialysis as judged by the larger amount of ^{65}Zn in the chamber with protein compared with the chamber containing buffer only (Table II). The multiple equilibria present in this test hamper quantitative treatment of the data, but qualitative conclusions can be drawn. HRG, although present at a much lower concentration (0.25 mg/mL) than the other proteins (up to 30 mg/mL), showed the greatest extent of ^{65}Zn binding and was able to significantly diminish the binding of Zn^{2+} by all other proteins at protein concentrations comparable to those found in serum. Unexpectedly, hemopexin, which binds a variety of heme and porphyrin analogues (Morgan, 1976), bound ^{65}Zn. However, the affinity of hemopexin for Zn^{2+} is low since HRG readily reduces the level of ^{65}Zn with hemopexin (Table II).

The proteins tested showed a variety of responses: hemopexin, extensive binding of ^{65}Zn but weak competition with HRG; albumin, extensive binding and strong competition; α_2-macroglobulin, minimal binding and strong competition; transferrin, minimal binding and weak competition. The reasons for this variety reflect the complexity found in serum, since binding alone is necessary but not sufficient to enable a protein to affect the dynamics of metal transport. Very tightly bound metals are not expected to dissociate sufficiently

Table II: Competition between Rabbit HRG and Other Serum Proteins for ^{65}Zn[a]

initial protein	% ^{65}Zn	competing protein	% ^{65}Zn
HRG	84	none	16[b]
	80	transferrin	20
	52	α_2-macroglobulin	48
	76	hemopexin	24
	52	albumin, 10 mg/mL	48
	48	albumin, 30 mg/mL	52
transferrin	56	none	44
	22	HRG	78
α_2-macroglobulin	55	none	45
	50	HRG	50
hemopexin	67	none	33
	27	HRG	73
albumin, 10 mg/mL	68	none	32
	58	HRG	42
albumin, 30 mg/mL	59	none	41
	55	HRG	45

[a] Experiments were conducted with an equilibrium dialysis apparatus as described in the text. ^{65}Zn was added at an initial concentration of 4.5 μM to the chamber containing the initial protein, and samples were taken from both chambers after 16 h. The buffer was 0.1 M Hepes and 0.25 M NaCl, pH 7.4. The protein concentrations were the following: rabbit HRG, 0.25 mg/mL (4.5 μM); rabbit hemopexin, 1 mg/mL; human serum albumin, 10 and 30 mg/mL; human transferrin, 2 mg/mL; human α_2-macroglobulin, 5 mg/mL. [b] With no competing protein present, the percent of ^{65}Zn in the second column reflects the percent of free ligand under these conditions.

either to alter the results of these experiments or to affect the transport of metals in serum. Once bound, such metals have a very low probability of dissociation and, like iron–transferrin, would require the action of receptors on target cells to effect release of the metal. The low extent of binding yet strong competition displayed by α_2-macroglobulin may arise from effects produced by endogenous zinc on this protein.

These results are being extended to include other metals and the effects of low molecular weight chelators. Several of these agents, e.g., citrate and certain amino acids, are found in serum and may influence metal dynamics in vivo. Although the relative affinities of the protein pairs used here will not be altered, the absolute distribution of metal may be. For example, under conditions similar to those in Table II, HRG shows a higher affinity for ^{65}Zn than does albumin even in the presence of physiological concentrations (0.1 mM) of L-histidine, but equilibrium is reached more rapidly, and over 10% of the ^{65}Zn is bound by the L-histidine (W. T. Morgan, unpublished experiments).

Conclusions

A major obstacle to interpretation of nearly all studies on the transport of metals such as Cu^{2+} and Zn^{2+} in serum, total concentration of each 15–20 μM (Sullivan et al., 1979), is that the protein-bound state of the metal is not evaluated or in many cases even considered. A particular problem is that direct, specific identifications of protein–metal complexes have not been made. For example, some workers define the zinc in serum that is not precipitated by poly(ethylene glycol) to be bound to albumin. This is clearly inadequate when dealing with such a complex mixture. In this laboratory, immunological methods are being developed to separate and identify protein–metal complexes in their natural milieu in serum.

HRG is able to bind a variety of divalent metals with high affinity in vitro, consistent with this protein having a role in the transport or homeostasis of metals in serum. Further work to explore this possibility is in progress with special emphasis on the physicochemical aspects of binding and on the physi-

ological actions of HRG in vivo.

Acknowledgments

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Physical Studies on the Ribosomal Protein S2 from the *Escherichia coli* 30S Subunit†

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ABSTRACT: The protein S2 has been isolated from the 30S subunit of *Escherichia coli* A19 ribosomes [Littlechild, J., & Malcolm, A. L. (1978) *Biochemistry 17*, 3363–3369]. This salt-extracted protein is soluble and does not aggregate at salt concentrations of 0.3–0.4 M as used under reconstitution conditions. This differs from the S2 protein extracted by the acetic acid and urea method. The molecular weight from sedimentation equilibrium was found to be 29 200, and the protein was found to have a $s^0_{20,w}$ value of 2.36 S. The apparent specific volume at 20 °C was 0.726 mL·g^{-1}, and the $D^0_{20,w}$ was 7.37×10^{-7} cm^2 s^{-1}. The value for intrinsic viscosity was found to be 6.42 mL·g^{-1}. An axial ratio of (5–6):1 for a prolate ellipsoid of revolution was estimated by using these parameters. The circular dichroism and proton magnetic resonance studies show that protein S2 has both substantial amounts of α helix and β-pleated sheet in solution and appears as a "folded" protein and not a random coil structure.

To understand the structural significance of the many components that make up the bacterial ribosome, it is essential to obtain information about the structure of individual ribosomal proteins. Such information is now available for many of the 53 different protein molecules as regards their secondary and tertiary structure and shape in solution [reviewed by Wittmann et al. (1979)].

The ribosomal protein S2 is the second largest protein in the 30S subunit of the *Escherichia coli* ribosome. Its molecular weight has been estimated from NaDodSO$_4$[1]–polyacrylamide gel electrophoresis to be 28 300 (Dzionara et al., 1970) and 29 000 (Littlechild & Malcolm, 1978), respectively. Values obtained from sedimentation studies vary from 24 000 (Dzionara et al., 1970) to 30 000 (Craven et al., 1969).

Neutron scattering experiments (Engelman et al., 1975) and immunological studies (Tischendorf et al., 1975) have both suggested that protein S2 has an elongated shape.

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[1] Abbreviations used: PhCH$_2$SO$_2$F, phenylmethanesulfonyl fluoride; BAM, benzamidine hydrochloride; NaDodSO$_4$, sodium dodecyl sulfate; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; CM, carboxymethyl; TP30, total protein extracted from the 30S ribosomal subunit with acetic acid according to Hardy et al. (1969); CD, circular dichroism.

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