Partial Characterization of the Heme-Binding Serum Glycoproteins Rabbit and Human Hemopexin*

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ABSTRACT: The molecular weight of rabbit hemopexin is 62,000 and that of human hemopexin 70,000, as determined by sedimentation equilibrium and by two polyacrylamide gel electrophoretic techniques. Twenty per cent of each molecule is carbohydrate including sialic acid, mannose, galactose, and glucosamine. No free SH groups can be detected; rabbit hemopexin has five and human hemopexin six S–S bridges.

The hemopexins, whether native, reduced, or reduced and carboxamidomethylated, fail to dissociate into subunits when examined in acrylamide gels at pH 9.4, 3.6, and 2.7, or in gels containing sodium dodecyl sulfate or high concentrations of urea. Neither do they dissociate when subjected to ultracentrifugation in 6 M guanidine hydrochloride. Therefore, they presumably consist of a single polypeptide chain.

Hemopexin, a serum β-glycoprotein, binds heme[1] with a greater affinity than albumin does (Nyman, 1960; Aber and Rowe, 1960; Muller-Eberhard and Liem, 1968). The heme-binding property of human hemopexin (HHx) was first described by Neale *et al.* (1958), who also observed the absence of this protein from sera of patients with hemolytic diseases. The following observations suggest that hemopexin is the main scavenger of heme and plays a role in the metabolism of heme and drugs: (a) hematin injected into humans (Sears, 1969) and into rabbits (Muller-Eberhard *et al.*, 1969a) causes a lowering of the serum hemopexin concentration, (b) the heme–hemopexin complex is eliminated by the liver parenchyma (Muller-Eberhard *et al.*, 1970), (c) known inducers of hepatic enzymes enhance hemopexin synthesis (Ross and Muller-Eberhard, 1970), and (d) the biological activity of one cytochrome diminishes coincidentally with removal of heme by hemopexin (Muller-Eberhard *et al.*, 1969b). A recent review stresses the biological implications of this protein (Muller-Eberhard, 1970).

HHx was characterized as a glycoprotein of approximately 80,000 molecular weight by Schultze *et al.* (1961), and the heme:protein ratio was found to be equimolar by Heide *et al.* (1964). More detailed information on HHx, in particular on its tendency to aggregate when highly purified, was reported by Muller-Eberhard and English (1967). The present report deals with a comparison of molecular weights, amino acid, and carbohydrate compositions of the hemopexins of two mammalian species, rabbit and human, and presents some investigations into the structure of these two proteins.

Experimental Section

Materials. RHx and HHx were purified from sera or Cohn fraction IV-7 (a gift of Cutter Laboratories) by precipitation with perchloric acid according to Schultze *et al.* (1961), and then followed by the technique of Muller-Eberhard and English (1967). Hemopexin prepared from human serum contained heme in a molar binding ratio of heme:protein of up to 0.1, that from Cohn fraction IV-7 had 0.3, and that from rabbit serum (apo-RHx) less than 0.03. Purity of the preparations was tested by polyacrylamide gel electrophoresis, as well as by immunoelectrophoresis and double diffusion in agar employing whole human and rabbit serum antibodies developed in goats. In all experiments, monomeric hemopexin was used exclusively. Protein concentrations were obtained either by the micro-Kjeldahl method, assuming for the protein an N content of 16%, or spectrophotometrically as described below.

Guanidine hydrochloride, Ultra Pure, was purchased from Mann Research Laboratories, and [^{14}C]*p*-mercuribenzoic acid (specific activity 10 mCi/mmole) from Calatomic. Urea (Mallinckrodt Chemical Works) was treated with Amberlite MB-1 ion exchanger and subsequently recrystallized from an ethanol–water mixture. Sodium dodecyl sulfate was obtained from Fischer Scientific Corp., and iodoacetamide from Mann Research Laboratories; the latter was recrystallized from water and used within 1 week.

Analytical Ultracentrifugation. Molecular weights were determined by the high-speed equilibrium method of Yphantis (1964), using a Spinco Model E instrument equipped with an ultraviolet absorption scanner. Sedimentation runs were performed in 0.1 M sodium phosphate (pH 7.1) at 20° for 18 hr, at 18,930 rpm; the base line was assessed by overspeeding to 44,000 rpm. The equation used for calculation of the molecular weights was $M = [2RT/(1 - \bar{V}\rho)w^2]\mathrm{d} \ln c/\mathrm{d}r^2$.

Polyacrylamide gel electrophoresis was carried out at pH 9.4 as described by Davis (1964); into some gels urea was incorporated to a final concentration of 8 M. Polyacrylamide gel electrophoresis was also employed, following the method of Reisfeld *et al.* (1962) at pH 3.6 and 2.7, employing β-alanine–acetic acid buffer. In experiments concerned with the electrophoretic homogeneity of the protein preparations (at pH 9.4), 100 μg of protein was applied; for subunit studies 25–50 μg was used.

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[1] Abbreviations used are: heme, ferriprotoporphyrin IX; RHx, rabbit hemopexin; HHx, human hemopexin; PAG, polyacrylamide gel; RCM, reduced and carboxamidomethylated; Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; PMB, *p*-mercuribenzoic acid.

TABLE I: Molecular Weights of RHx and HHx as Determined by Sedimentation Equilibrium and Polyacrylamide Gel Electrophoresis.

Protein	Ultracentrifugation[a]	Polyacrylamide Gel Electrophoresis[b]	Polyacrylamide Gel Electrophoresis[c]	Av Mol Wt
RHx	57,000	64,500	64,000	61,800
HHx	69,800	72,000	68,000	69,800

Methods of: [a] Yphantis (1964). [b] Hedrick and Smith (1968). [c] Shapiro *et al.* (1967).

TABLE II: Molecular Weights of Oligomeric States of HHx and RHx as Determined by Polyacrylamide Gel Electrophoresis.[a]

HHx			RHx	
Mol Wt	Mol Wt 69,800	Oligomeric State	Mol Wt	Mol Wt 61,800
72,000	1.03	Monomer	66,000	1.07
119,000	1.71	Dimer	117,000	1.89
180,000	2.56	Trimer	203,000	3.29

[a] Method of Hedrick and Smith (1968).

For determination of the molecular weight the procedure of Hedrick and Smith (1968) was followed, but gel and buffer compositions of Davis (1964) were used. Hemopexin (50 μg) was layered onto each of 10 gels containing 3.75–10.5% acrylamide and the respective R_F values were measured in reference to the dye front. The molecular weights of the hemopexins were derived from the calibration curve relating the parameter, S, and the log (molecular weight), where $S = \Delta 100 \ln R_F/\Delta$ gel concentration. For calibration, four proteins of known molecular weight were used: horse myoglobin (17,500); chicken ovalbumin (43,000); bovine serum albumin monomer (68,000), dimer, and trimer; and human transferrin monomer (74,000), dimer, and trimer.

In addition, polyacrylamide gel electrophoresis was carried out according to Shapiro *et al.* (1967) in 5.25% gels containing 0.1% SDS at pH 7.0. The molecular weights of the hemopexins were obtained from a plot of log molecular weight *vs.* R_F. In addition to the proteins mentioned above, pepsin (34,000); human IgG L chain (20,000); and H chain (57,000) were also used as standards. The R_F values were calculated from the migration distance of the protein compared to that of pepsin admixed as a reference.

Amino Acid Analysis. The amino acid content was determined by the technique of Spackman *et al.* (1958), employing a Beckman 120B instrument. Proteins were hydrolyzed according to Moore (1963) for 24 and 72 hr. The values for serine and threonine were taken from the 24-hr hydrolysates and those for lysine, histidine, arginine, valine, isoleucine, leucine, and phenylalanine were taken from the 72-hr hydrolysates. Values derived from both hydrolysates were averaged to obtain the content of the remaining amino acids. Following the procedure of Moore (1963), half-Cys and methionine of performic acid oxidized proteins were determined as cysteic acid and methionine sulfone, respectively. Tryptophan content was determined on RCM protein utilizing the spectrophotometric methods of Bencze and Schmid (1957) and Edelhoch (1967).

Reduction and Carboxamidomethylation. Proteins were treated with 6 M Gdn·HCl and 0.15 M β-mercaptoethanol at pH 7.5 in 0.4 M Tris-glycine for 3 hr at 37° under N_2. Iodoacetamide was added in 10-fold excess over β-mercaptoethanol, and the reaction mixture was left at room temperature for 1 hr prior to dialysis in 0.2 M Tris-glycine (pH 8.3) with or without 8 M urea.

Assessment of SH Groups. Radioactively labeled PMB was used for the determination of SH groups. A 50 molar excess of PMB was added to 4 moles of both hemopexins in 0.05 M sodium phosphate buffer (pH 7.5), with or without Gdn·HCl (final concentration 6 M). After 20 hr at room temperature, the reaction mixtures were exhaustively dialyzed for 1 week in 0.01 M sodium phosphate (pH 7.5). Between 150 and 320 μg of protein (concentration determined spectrophotometrically) in 1 ml were counted with a Beckman low β counter background on stainless steel planchets (size 2 in. × 1/8 in.). The standards used were 250 μg of protein in 1 ml of 0.01 M sodium phosphate containing PMB equivalent to one SH group per mole of protein.

Absorption Spectrophotometry. Ultraviolet and visible spectra of RHx and HHx were recorded on a Cary 14 recording spectrophotometer. Extinction coefficients and the exact position of the absorption maxima were obtained with a Zeiss PMQ II spectrophotometer.

Results and Discussion

Molecular Weights. Results obtained by ultracentrifugation and by polyacrylamide gel electrophoresis are shown in Table I. The ultracentrifugal data represent an average of two experiments, whereas polyacrylamide gel electrophoresis data are an average of five or more determinations. The $\bar{v}$, 0.725, used in the calculation of the ultracentrifugal data was derived from the amino acid content not considering the carbohydrate content of the protein (McMeekin *et al.*, 1949). The molecular weight of HHx is 10,000 lower than that reported by Schultze *et al.* (1961), who estimated their value from the sedimentation coefficient. The reason for the difference in data obtained for RHx by ultracentrifugation and by polyacrylamide gel electrophoresis is presently unknown but under investigation.

In Table II, the molecular weights were given for several oligomeric states of HHx and RHx as obtaine dby polyacrylamide gel electrophoresis analysis. Aggregation ensued when the protein solutions were exposed to 60° for at least 60 min and when left at 4° for several days. Saturation with heme did not prevent polymerization. Extensive polymerization of HHx and RHx occurs also during the purification procedure as described for HHx (Muller-Eberhard and English, 1967), and is the main reason for the low yield of purified protein. However, exposure to 6 M urea produces monomers.

Amino acid and carbohydrate composition of the two hemopexins are presented in Tables III and IV. Amino acid contents were calculated for a molecular weight of 49,350 for RHx and 54,750 for HHx, which represent the

TABLE III: Amino Acid Composition of RHx and HHx.

Amino Acid Residue	RHx g/100 g of Peptide	RHx No. of Residues/ 10^5 g of Peptide	RHx No. of Residues[a]/ Molecule	RHx Nearest-Integral No./ Molecule	HHx g/100 g of Peptide	HHx No. of Residues/ 10^5 g of Residues	HHx No. of Residues[b]/ Molecule	HHx Nearest-Integral No./ Molecule
Lys	5.88	45.9	22.7	23	6.03	47.0	25.7	26
His	4.86	35.5	17.4	17	4.90	35.7	19.5	20
Arg	7.17	45.9	22.7	23	6.46	41.4	22.7	23
Asp	10.56	91.8	45.3	45	9.74	84.7	46.4	46
Thr	4.85	48.0	23.7	24	4.56	45.1	24.7	25
Ser	5.63	64.7	31.9	32	5.08	58.3	31.9	32
Glu	9.97	77.2	38.1	38	10.69	82.8	45.3	45
Pro	5.68	58.4	28.8	29	6.58	67.7	37.1	37
Gly	4.88	85.5	42.2	42	5.15	90.3	49.4	49
Ala	3.71	52.2	25.8	26	4.28	60.2	33.0	33
Cys/2[c]	2.15	20.9	10.3	10	2.33	22.6	12.4	12
Val	5.17	52.2	25.8	26	5.04	50.8	27.8	28
Met[d]	1.10	8.3	4.1	4	1.48	11.3	6.2	4
Ile	2.60	23.0	11.3	11	2.13	18.8	10.3	10
Leu	8.50	75.1	37.1	37	8.52	75.2	41.2	41
Tyr	4.77	29.2	14.4	14	4.91	30.1	16.4	16
Phe	5.53	37.6	18.6	19	5.81	39.5	21.6	22
Trp[e]	5.99	37.6	18.5	18	6.31	33.9	18.5	18
Total	100.00	889.0	438.7	438	100.00	895.4	490.1	489

[a] Calculated for a molecular weight of 49,350 (79.84% of 61,800 molecular weight, the average molecular weight, Table I). [b] Calculated for a molecular weight of 54,750 (78.44% of 69,800 molecular weight, the average molecular weight, Table I). [c] Determined as cysteic acid on performic acid oxidized protein. [d] Determined as methionine sulfone on performic acid oxidized protein. [e] Determined spectrophotometrically.

TABLE IV: Carbohydrate Composition of RHx and HHx.

Carbohydrate Residues	RHx g/100 g of Protein	RHx No. of Residues/ Molecule[a]	RHx Nearest-Integral No. of Residues	HHx g/100 g of Protein	HHx No. of Residues/ Molecule[b]	HHx Nearest-Integral No. of Residues
Mannose[c]	2.32	8.8	9	3.84	16.5	17
Galactose[c]	1.76	6.7	7	3.08	13.3	13
Glucosamine[d]	8.21	26.8	27	7.73	28.6	29
N-Acetylneuraminic acid[e]	7.87	18.6	19	6.92	18.5	18
Total	20.16	60.9	62	21.57	76.9	77
Calculated molecular weight			12,657			15,043

[a] Calculated for a molecular weight of 12,450 corresponding to the carbohydrate portion of the RHx molecule (20.16% of 61,800 molecular weight). [b] Calculated for a molecular weight of 15,050 corresponding to the carbohydrate portion of the HHx molecule (21.56% of 69,800 molecular weight). [c] Performed kindly by Dr. R. J. Winzler (Lehnhardt and Winzler, 1968). [d] Kindly performed by Dr. C. A. Abel (Abel *et al.*, 1968). [e] Kindly performed by Dr. E. H. Eyler (Svennerholm (1957) and Warren (1959)).

average molecular weights determined by ultracentrifugation and polyacrylamide gel electrophoresis (Table I), minus the carbohydrate content. The number of SH groups for RHx with and without Gdn·HCl were 0.15 and 0.19 per molecule, respectively, and those for HHx 0.19 and 0.25. Therefore, it can be concluded that all 10 half-Cys of RHx and all 12 half-Cys of HHx form S–S bridges. Both proteins contained the same carbohydrates, mannose, galactose, glucosamine, and sialic acid, which accounted for approximately 20% of the total molecular weight. The amino acid composition of HHx is almost identical with that reported by Heimburger *et al.* (1964).

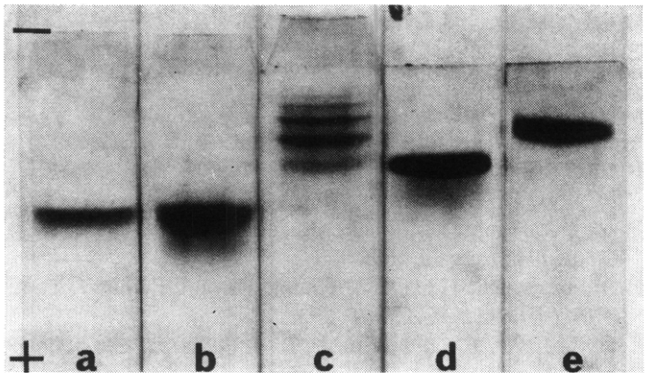

FIGURE 1: Electrophoresis of RHx in 7.5% polyacrylamide gels, pH 9.4 Tris-glycine (a–c); gels d and e also contained 8 M urea. The following samples were applied: (a) native RHx, (b and d) RHx treated for 3 hr with 6 M Gdn·HCl, and (c and e) RHx which was reduced and carboxamidomethylated in 6 M Gdn·HCl.

Effect of Gdn·HCl and SDS. Experiments discussed in this section indicate that a single polypeptide chain is the structural unit of the hemopexins. The reduced and RCM forms of RHx and HHx were analyzed in the presence of SDS by polyacrylamide gel electrophoresis according to Shapiro *et al.* (1967). Only one band was observed in each instance, and the molecular weight of the modified proteins did not significantly differ from that of the unmodified proteins (Table V).

The hemopexins were also reduced and carboxamidomethylated in 6 M Gdn·HCl at pH 7.5 and examined by polyacrylamide gel electrophoresis in gels with and without 8 M urea. In Figure 1, a representative experiment is shown. The first three experiments (a–c) were analyzed in regular gels, the latter two (d and e) in gels with 8 M urea. The unmodified protein was applied in gel a, the Gdn·HCl-treated protein in gels b and d, and, in addition, the RCM protein in gels c and e. The migration of both monomeric and Gdn·HCl-treated RHx was identical in gels with or without urea (control in urea not shown). The RCM protein, however, resolved into several bands of slower mobility than that of the monomer (gel a). Reduction and carboxamidomethylation caused polymerization which was reversed by 8 M urea (gel e).

Further evidence that reduction of the disulfide bonds does not result in subunit formation was obtained by ultracentrifugation. Sedimentation equilibrium was performed on HHx in 6 M Gdn·HCl and 0.01 M dithiothreitol.[2] The molecular weight of the protein thus treated was 67,500; this value corresponds to the molecular weight of the unmodified monomer (Table I). Attempts to dissociate hemopexin into subunits at low pH were also unsuccessful. Aggregation, rather than dissociation, was found in polyacrylamide gel electrophoresis at pH 3.6 and 2.7. Thus treatment with urea, Gdn·HCl, and SDS as well as exposure to low pH does not yield subunits of the hemopexins. Moreover, the aggregation enhanced by reduction and carboxamidomethylation is readily reversible in urea.

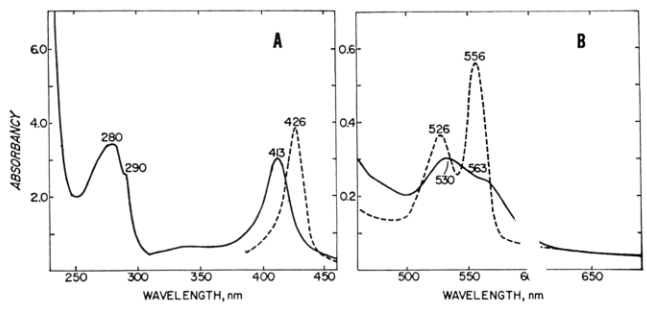

FIGURE 2: Near-ultraviolet and visible spectrum of RHx in 0.05 M phosphate (pH 7.1). Protein concentration 1.3 mg/ml, *i.e.*, at 21 μM solution. Hematin, prepared as previously described (Muller-Eberhard *et al.*, 1969a), was added to the apo-RHx to a 1.0 M ratio. Spectra from wavelengths 240 to 460 mμ in panel A, and that from 460 to 770 mμ in panel B. Heme–hemopexin (———), reduced with dithionite (- - - - - - -).

TABLE V: Comparison of Molecular Weights[a] of Reduced and RCM RHx and HHx in Polyacrylamide Gel Electrophoresis Containing SDS.

Treatment	RHx	HHx
1% SDS	59,000	64,000
1% SDS + reduced	64,000	68,000
1% SDS + RCM	66,000	70,000

[a] Shapiro *et al.* (1967).

Absorption Spectra. Both hemopexins when saturated with heme show several absorption maxima in the ultraviolet and visible regions. The spectrum of RHx, nearly identical with that of HHx (Heide *et al.*, 1964), is depicted in Figure 2. The Soret absorption, as in other heme proteins, has a greater intensity than that of the other maxima in the visible region. Addition of dithionite increased the extinction of the Soret band which was shifted to a longer wavelength. In addition, the broad peak in the visible region resolved into an α and a β band. After removal of excess of reducing reagent by dialysis or by passing the reduced protein over a Sephadex G-25 column, spontaneous reoxidation occurred and the original spectrum was restored. These findings resemble those characteristic for the *c*-type cytochrome (Margoliash and Schejter, 1966).

Spectrophotometric titration of apo-RHx and heme-poor HHx with heme was performed at the wavelengths corresponding to absorption maxima in the Soret region, *i.e.*, for RHx at 413.5 nm and for HHx at 414 nm in 0.025 M sodium borate (pH 9.1). As previously described for HHx by Heide *et al.* (1964), addition of increasing amounts of heme to RHx resulted in an abrupt change in the slope of the titration curve at an equimolar ratio of heme to protein. On further addition of heme, the slope followed that of heme solution. Upon saturation with heme, the extinction at 280 nm was approximately 10% higher than that of the unsaturated hemopexin.

The extinction coefficients derived from these experiments are listed in Table VI. Identical values for the $E_{1\,cm}^{1\%}$ were observed for 0.05 M sodium phosphate (pH 7.1) and for 0.025 M sodium borate (pH 9.1). Measurement of the extinction at 280 nm and at the maximum in the Soret region permits determination of the concentration of hemopexin as

[2] Kindly performed by Dr. J. E. Blair, Institute of Enzyme Research, Madison, Wis., according to the method by Blair *et al.* (1968). In the calculations, the apparent specific volume was assumed to be the same as that for the native HHx.

TABLE VI: Extinction Coefficients[a] of RHx and HHx.

Mole of Heme/Mole of Hx	Wavelength (nm)	$E^{1\%}_{1\text{ cm}}$ RHx	$E^{1\%}_{1\text{ cm}}$ HHx
0	280	23.9	23.8
1	280	26.4	26.4
1	413.5	23.2	
1	414		23.0

[a] Same in 0.05 M phosphate (pH 7.1) and in 0.025 M sodium borate (pH 9.1).

well as of the molar ratio of heme to protein. The value for HHx, 23.8, at 280 nm is considerably higher than that, 16.9, reported by Schultze *et al.* (1961).

In conclusion, HHx and RHx contain similar carbohydrate moieties which comprise approximately equivalent portions of the molecule. They differ in molecular weight but appear both to consist of only one polypeptide chain which binds an equimolar amount of heme.

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