An Avian Serum α_1-Glycoprotein, Hemopexin, Differing Significantly in both Amino Acid and Carbohydrate Composition from Mammalian (β-Glycoprotein) Counterparts†

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ABSTRACT: We report here on physicochemical characteristics of chicken hemopexin, which can be isolated by heme–agarose affinity chromatography [Tsutsui, K., & Mueller, G. C. (1982) *J. Biol. Chem. 257*, 3925–3931], in comparison with representative mammalian hemopexins of rat, rabbit, and human. The avian polypeptide chain appears to be slightly longer (52 kDa) than the human, rat, or rabbit forms (49 kDa), and also the glycoprotein differs from the mammalian hemopexins in being an α_1-glycoprotein instead of a β_1-glycoprotein. This distinct electrophoretic mobility probably arises from significant differences in the amino acid composition of the chicken form, which, although lower in serine and particularly in lysine, has a much higher glutamine/glutamate and arginine content, and also a higher proline, glycine, and histidine content, than the mammalian hemopexins. Compositional analyses and ^{125}I concanavalin A and ^{125}I wheat germ agglutinin binding suggest that chicken hemopexin has a mixture of three fucose-free N-linked bi- and triantennary oligosaccharides. In contrast, human hemopexin has five N-linked oligosaccharides and an additional O-linked glycan blocking the N-terminal threonine residue [Takahashi, N., Takahashi, Y., & Putnam, F. W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2021–2025], while the rabbit form has four N-linked oligosaccharides [Morgan, W. T., & Smith, A. (1984) *J. Biol. Chem. 259*, 12001–12006]. In keeping with the finding of a simpler carbohydrate structure, the avian hemopexin exhibits only a single band on polyacrylamide gel electrophoresis under both nondenaturing and denaturing conditions, whereas the hemopexins of the three mammalian species tested show several bands. In contrast, the isoelectric focusing pattern of chicken hemopexin is very complex, revealing at least nine bands between pH 4.0 and pH 5.0, while the other hemopexins show a broad smear of multiple ill-defined bands in the same region. Like its mammalian counterparts, chicken hemopexin resists digestion by trypsin and plasmin when saturated with heme. Each of the four hemopexins forms a distinct peptide map on sodium dodecyl sulfate–polyacrylamide gel electrophoresis following tryptic digestion. These results indicate that the hemopexin of avians differs substantially from the hemopexins of mammals, which, as a group, show a notable similarity with regard to carbohydrate structure and amino acid composition.

The β-glycoprotein hemopexin (Hx),[1] an important plasma component in all mammalian species, functions in the disposal of intravascular heme (Muller-Eberhard & Liem, 1974). Hx binds heme with high affinity (Hrkal et al., 1980) and releases it from the circulating heme–Hx complex at the hepatic sinusoidal membrane (Smith & Morgan, 1981); within the liver parenchymal cells the heme is degraded to bilirubin (Davies et al., 1979) and the iron conserved. Hx interacts with heme in an equimolar ratio (Heide et al., 1964) and exhibits an affinity for heme higher than that shown by methemoglobin subunits (Hrkal et al., 1974). This protein is produced in liver cells and, although highly glycosylated (~20% by weight), is secreted soon after its synthesis (Katz et al., 1985).

Recently, in collaboration with Dr. Gerd Grieninger of the New York Blood Center, we established the identity of chicken Hx and α_1-globulin M, a plasma protein whose synthesis is greatly responsive to hormonal supplementation in monolayer cultures of chicken embryo hepatocytes maintained in serum-free, defined medium (Grieninger et al., 1986). This finding suggests that the synthesis of Hx may be under hormonal regulation in all species. The alteration seen in serum hemopexin levels in various mammals in response to agents such as carcinogens, porphyrinogens, acute-phase stimulants, and heme [Foidart et al., 1982; references cited in Muller-Eberhard and Liem (1974)] may be in part secondary to regulation by hormones.

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[1] Abbreviations: Hx, hemopexin; heme, iron protoporphyrin IX (regardless of oxidation state); BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS (–PAG), sodium dodecyl sulfate (–polyacrylamide gel); P:NGase, *N*-glycanase; Endo H, endo-β-*N*-acetylglucosaminidase H; ConA, concanavalin A; WGA, wheat germ agglutinin; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; NeuNAc, *N*-acetylneuraminic acid (sialic acid); STI, soybean trypsin inhibitor; CD, circular dichroism; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high-performance liquid chromatography; SE, standard error; IEF, isoelectric focusing; PEG, poly(ethylene glycol); PMSF, phenylmethanesulfonyl fluoride.

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Since previous attempts to purify chicken Hx by the methods commonly used for purifying Hx from mammalian plasmas (Hrkal & Muller-Eberhard, 1971; Vretblad & Hjorth, 1977) were unsuccessful, and because it was observed that no plasma protein of chickens cross-reacted with antisera raised against human or rabbit Hx (Cox et al., 1978), it had been assumed that Hx, if it existed in birds at all, must differ greatly in structure from Hx in mammals. We have persisted in seeking to prove the existence of Hx in avians,[2] as its absence would have made it questionable that hemopexin is a pivotal protein for heme transport. The presence of hemopexin in chicken plasma was verified, and it was found to be an α_1-glycoprotein.

In this paper we report on some physicochemical and immunological properties of chicken Hx, significantly different from those of the mammalian Hxs to which it is compared, and we present as well the details of an isolation technique employing affinity chromatography on heme–agarose (Tsutsui & Mueller, 1982a).

Materials and Methods

Synthesis of Heme–Agarose. Heme, as hemin chloride (Sigma, type I), was linked to ω-(aminoethyl)agarose (Sigma) according to the method of Tsutsui and Mueller (1982a). In spite of thorough washing with 25% pyridine, traces of heme still leaked from the heme–agarose column to which plasma or serum was applied, significantly reducing the yield of purified hemopexin. To remove unbound heme, we first incubated the newly prepared heme–agarose with approximately 200 mL of plasma or serum for 2 h at room temperature. After initial washing with 10 mM sodium phosphate buffer, pH 7.5, and 500 mM NaCl and elution of absorbed proteins with 200 mM acetic acid, the heme–agarose was again washed and equilibrated in buffer, with NaN_3 added to 0.04% final concentration, and stored in a dark bottle at 4 °C.

Isolation of Chicken Hx. The isolation procedure for chicken Hx was a slight modification of the method of Tsutsui and Mueller developed for the purification of rabbit Hx (Tsutsui & Mueller, 1982b). One hundred milliliters of citrated chicken plasma (purchased from Hazelton Dutchland Inc., Denver, PA) was quickly thawed at 56 °C, and after the addition of 25 μg of aprotinin/mL and 10^{-4} M phenylmethanesulfonyl fluoride (PMSF; Sigma), the mixture was cooled on ice for 1 h and filtered through a funnel plugged with glass wool. The filtered plasma was dialyzed against 10 mM sodium phosphate buffer, pH 7.5, and 1 M NaCl and was then mixed with 100 mL of heme–agarose.

The binding of chicken Hx to heme–agarose was performed in darkness with slow stirring for 2 h at room temperature, or overnight at 4 °C. The heme–agarose was washed thoroughly in a fritted glass filter funnel with approximately 1 L of 10 mM sodium phosphate, pH 7.5, 100 mM NaCl, 2.5 μg/mL aprotinin, and 10^{-5} M PMSF. Bound protein was eluted with 200 mL of 50 mM succinic acid, pH 2.8, and 500 mM NaCl, concentrated on Amicon Diaflo membrane PM 10, and then dialyzed overnight at 4 °C against 20 mM sodium acetate, pH 5.2, 50 mM NaCl, and 0.02% NaN_3. The precipitate formed during dialysis was removed by centrifugation at 10000*g* for 10 min at 4 °C in a Beckman Model J-21C centrifuge and the supernatant applied to a SP-Sephadex column (3 × 16 cm) equilibrated at 4 °C with 3 volumes of the dialysis buffer. A 50–250 mM linear NaCl gradient (500-mL total volume) was applied. Chicken Hx eluted at 80–100 mM NaCl whereas that of other species (human, rat, and rabbit) eluted at 180–200 mM NaCl.

Fractions containing chicken Hx were concentrated and dialyzed against 50 mM sodium phosphate, pH 7.5, and 0.1 M NaCl; aprotinin and PMSF, 2.5 μg/mL and 10^{-5} M respective final concentrations, were added.

Protein concentration was determined by the method of Bradford (1976), using twice-crystallized bovine serum albumin (BSA; Sigma) as a standard protein, and by spectrophotometric methods (Seery et al., 1972) on a Beckman DU spectrophotometer (Model 2400).

Gel Electrophoresis. Polyacrylamide gel (PAG) electrophoresis in a nondenaturing system was carried out according to the procedure provided by Sigma Chemical Co. (Technical Bulletin No. MKR-137), based on modified methods of Bryan (1977) and Davis (1964). The molecular weight for chicken Hx was determined by using modified Ferguson plots described in the technical bulletin. Protein standards for the molecular weight range 14000–480000 were from Sigma.

The purity and the relative molecular weight of the Hxs were established by electrophoresis performed according to Laemmli (1970) and with 7.5–15% gradient sodium dodecyl sulfate (SDS)–polyacrylamide gels (PAGs) (1.5-mm thickness). Gels were stained in a solution containing final concentrations of 0.1% Coomassie blue, 25% 2-propanol, and 10% acetic acid and destained by diffusion in a solution of 10% 2-propanol and 10% acetic acid. All electrophoresis reagents and protein standards were from Bio-Rad. After electrophoresis of radiolabeled Hx, gels were destained, rinsed for 15 min in water, and soaked for 1–2 h in Autofluor (National Diagnostics, Somerville, NJ). Gels were dried on a gel slab dryer (Bio-Rad) and exposed for autoradiography to Kodak X-Omat RP films at −70 °C.

Isoelectric Focusing. Isoelectric focusing was performed on ampholine PAG plates (LKB) using MultiPhor II 2117 (LKB). Protein standards in the range of pH 2.5–6.5 were from Pharmacia (Piscataway, NJ). Initially, isoelectric focusing was carried out by using the pH range 3.5–9.5 and was subsequently performed by using ampholine PAG plates with a narrow pH range (4.0–5.0). After isoelectric focusing was complete, the pH of the gel strips was measured.

Gel Filtration. Gel filtration of chicken and rat Hx was performed on an Ultrogel AcA44 column (LKB) (1.6 × 74 cm) equilibrated and eluted with 10 mM Tris-HCl, pH 7.4, and 0.1 M NaCl at a flow rate of 10 mL/h. Dextran blue (M_r 2000000) and the following protein standards were from Pharmacia: BSA (M_r 67000), ovalbumin (M_r 43000), chymotrypsinogen A (M_r 25000), and ribonuclease A (M_r 13700).

Spectrophotometric and Fluorometric Analyses. Heme (hemin chloride) was dissolved in Me_2SO and further diluted in 40% Me_2SO. Concentrations were measured spectrophotometrically as the pyridine hemochrome (Falk, 1964). Heme binding to chicken Hx was titrated in 50 mM sodium phosphate and 100 mM NaCl, pH 7.5, by recording absorption at 414 nm in a Cary 118C spectrophotometer.

Binding of mesoheme and bilirubin (both from Porphyrin Products, Logan, UT) to Hx was determined fluorometrically on a Perkin-Elmer MPF 44A fluorescence spectrophotometer. The concentration of mesoheme was determined by the same method as that used for heme (Falk, 1964), and the bilirubin concentration was measured in 50 mM NaOH spectrophotometrically (Blauer & King, 1970). The ligands were added to the protein until the tryptophan emission spectrum was completely quenched.

Immunological Characteristics. Immunoelectrophoresis in 2% agarose was performed by the method of Scheidegger

[2] Recently we discovered reference to pheasant Hx in Baker et al. (1966).

(1955). Details are described in the legend to Figure 1. The level of chicken Hx in plasma and serum was measured by rocket immunoelectrophoresis (Laurell, 1966). We added 5% poly(ethylene glycol) (PEG) 4000 (w/v) and 0.5 mM calcium lactate (final concentration) to the agarose to enhance precipitation (Plumley & Schmidt, 1983) and used specific antibodies, raised in rabbits, purified by affinity chromatography on Hx coupled to CNBr-activated Sepharose 4B (Sigma).

Western blots (Burnette, 1981) were performed as follows: The samples were electrophoresed on 7.5–15% gradient SDS–PAG electrophoresis and the gels removed and equilibrated for 15 min in 25 mM Tris buffer, pH 8.3, containing 200 mM glycine and 20% methanol. The proteins were transferred onto nitrocellulose filters by electrophoresis for 1 h at 4 °C using the same Tris buffer. The nitrocellulose filters were then incubated first in blocking solution [5% dried milk (w/v), 10 mM Tris–saline, 0.1% NP-40, and 0.02% NaN_3] for 1 h and then for 2 h at room temperature in blocking solution containing specific antibodies raised in rabbit or mouse. After being washed with blocking solution, the filters were incubated with either ^{125}I goat anti-rabbit IgG or ^{125}I rabbit anti-mouse IgG for 1 h at room temperature. Filters were then washed extensively with phosphate-buffered saline (PBS) containing 0.2% Tween 20, and the bands were visualized by autoradiography.

Amino Acid Analysis. Amino acid analysis of chicken Hx was performed in two different laboratories by using three different preparations of the protein.

(A) The following procedure was used in the laboratory of R.B.T.: After exhaustive dialysis in water, duplicate samples of chicken Hx (0.25 nmol) were hydrolyzed in N_2-flushed vials in vacuo with 1 mL of redistilled 6 N HCl for 22, 46, and 70 h at 108 °C. Norleucine was added as an internal standard prior to hydrolysis, and samples were analyzed on a Beckman 119CL amino acid analyzer. Values for serine and threonine were obtained by extrapolation to zero hydrolysis time, while the values for glycine, alanine, valine, and isoleucine were those measured at 70 h. Tryptophan content was calculated by the method of Edelhoch (1967) from spectrophotometric measurements and by using the tyrosine value determined by analysis. Cysteine was determined as cysteic acid after oxidation with Me_2SO by the method of Spencer and Wold (1969). Recoveries, based on norleucine, were routinely 96–99%. An $E^{1\%}_{1cm}$ value of 20.5 at 280 nm was established for the protein moiety of chicken Hx on the basis of quantitative amino acid recovery.

(B) The following procedure was used in the laboratory of D.W.: Triplicates of two different preparations of chicken and rat Hx were analyzed as follows: The proteins were hydrolyzed in sealed evacuated tubes in 6 N HCl at 110 °C for 24, 48, and 72 h. The solutions were then evaporated to dryness, and the residue was redissolved in water and applied to a Durrum D-500 amino acid analyzer. The values were averaged for the three hydrolysis times, except for serine and threonine which were extrapolated to zero hydrolysis time and valine and isoleucine for which the 72 h value was used. The weight of protein analyzed was calculated by summing the residue weights of the amino acids found and adding the amount of tryptophan determined spectrophotometrically (Edelhoch, 1967).

Carbohydrate Analysis. Neutral sugars (mannose, galactose, glucose, fucose) were determined in duplicate samples (3.2 nmol) of the chicken Hx dialyzed for amino acid analyses. Samples were hydrolyzed in argon-flushed vials in vacuo with 1 mL of 2 N HCl for 2.5 and 4 h at 100 °C. Rhamnose was added as an internal standard prior to hydrolysis, and samples were analyzed by chromatography on a Dowex 1X4 (10–20 μm) column (0.6 × 23 cm) by a modification of the method of Honda et al. (1974). The column was maintained at 60 °C and eluted at 0.8 mL/min sequentially for 45 min with 0.3 M sodium borate, pH 7.4, for 60 min with 0.6 M sodium borate, pH 7.0, and finally, for 35 min with 1 M sodium borate, pH 7.0. The column effluent was mixed with the detection reagent consisting of 0.3 M ethylenediamine, 0.5 M H_3BO_4, and 0.5 M H_3PO_4, pH 5.0, at a flow rate of 0.4 mL/min and heated in a reaction coil at 140 °C for 6.4 min before passing through a Waters 420-AC fluorescence detector. A 360-nm band-pass filter was used for excitation, and a 425-nm long-pass filter used for detection. Recovery of neutral sugars, based on rhamnose, was 96–98%.

N-Acetylglucosamine (GlcNAc) was determined as glucosamine in duplicate samples (0.4 nmol) of the dialyzed Hx hydrolyzed in argon-flushed vials in vacuo with 1 mL of 2 N HCl for 18 h at 108 °C. Glucosaminitol was added as an internal standard, and samples were analyzed on Beckman PA-35 resin by using a Beckman 119CL amino acid analyzer (Plummer, 1976). Recoveries, based on glucosaminitol, were 98%. *N*-Acetylgalactosamine (GalNAc), which elutes on PA-35 in the position of glucosaminitol, was not found in control hydrolysates.

Sialic acid (NeuNAc) was determined on samples (0.25 nmol) of dialyzed chicken Hx hydrolyzed for 35 min at 80 °C with 0.05 M H_2SO_4 by the 0.8-mL microassay described by Skoza and Mohos (1976).

Glycosidase Digestions. Chicken Hx (10 μg) was digested by treatment with peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase (P:NGase F), 2.4 milliunits, from cultural filtrates of *Flavobacterium meningosepticum* (Tarentino et al., 1985) in a final volume of 100 μL containing 150 mM sodium phosphate buffer, pH 8.6, or by treatment with endo-β-*N*-acetylglucosaminidase H (Endo H), 4 milliunits [purified from pkCE3-transformed *Escherichia coli* (Trumbly et al., 1985)], in a final volume of 100 μL containing 150 mM sodium citrate buffer, pH 5.5. Hx was first denatured by heating at 100 °C for 3 min in the presence of 0.1% SDS and then cooled to room temperature. Triton X-100 added to 0.7% to complex the unbound SDS as mixed micelles. The glycosidases were added to separate samples, and digestion was allowed to proceed for 18 h at room temperature. Reactions were terminated by the addition of 80 μL of SDS- and β-mercaptoethanol-containing Laemmli sample buffer (Laemmli, 1970) to each mixture. The samples were heated for 4 min at 100 °C and 10-μL aliquots subjected to SDS–PAG electrophoresis.

Gel Electrophoresis and ^{125}I-Lectin Overlay. Hx samples, untreated or treated with glycosidases, were electrophoresed in 0.75-mm-thick SDS–acrylamide slab gels according to Laemmli (1970). The gels were 11% monomer [acrylamide:bis(acrylamide) = 42.5:1]; the stacking gel was 4.5%. Gels were stained with Coomassie blue R-250, destained, and stored in 10% acetic acid containing 10% ethanol.

After photography, gels were equilibrated with binding buffer and overlayed with ^{125}I-ConA or ^{125}I-WGA for 8 h as described (Chu et al., 1981) except that the carrier hemoglobin was present at 2 mg/mL. Gels were washed for 4 days in binding buffer plus NaN_3, dried, and exposed to Kodak X-Omat AR film at −80 °C. The concanavalin A (ConA) and wheat germ agglutinin (WGA) lectins (Boehringer Mannheim) were iodinated by a modification (Chu et al., 1981) of the mild two-stage procedure of Syvanen et al. (1973), which

Table I: Yield of Hx Purified from Plasma of Chicken and Rat

purification step	yield (mg) chicken Hx[a]	rat Hx[a]
plasma (100 mL)	108 ● 42	178 ± 9
heme–agarose	54.7 ● 5.3	58 ± 8.48
SP-Sephadex	39.4 ± 13.0	34 ● 7.6
recovery (%)	36.5	19.1

[a] Mean value ± SE from three separate experiments.

employs Na^{125}I (Amersham) and chloramine T (Fisher Scientific).

Proteolytic Degradation of Hemopexin. Human plasminogen (Sigma) was activated by urokinase from human kidney cells (Sigma) according to the method of Robbins et al. (1967), modified by Rickli and Otavsky (1975). Urokinase, 5 units, was added to 5 units of plasminogen (dissolved in 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and 25% glycerol), and the activation was allowed to proceed at room temperature for 24 h.

The optimal concentration of plasmin needed to digest 15 μg of Hx was found by incubation of apo-Hx with various concentrations of plasmin in the range of 0.01–0.25 unit. After incubation, the reaction was terminated by addition of Laemmli sample buffer. The samples were boiled for 1 min and immediately applied onto 7.5–15% SDS–PAG. For details of the digestion of apo-Hx and heme–Hx with plasmin, see the legend to Figure 6.

For trypsin digestion analysis, 100 μg of Hx was labeled with 100 μCi of ^{125}I (New England Nuclear) by the chloramine T method (Greenwood et al., 1963). To each sample (equivalent to about 500 000 cpm) of ^{125}I-Hx was added 15 μg of unlabeled protein and 5 μg of trypsin (Worthington Biochemical Corp.). For details of the digestion of apo-Hx and of heme–Hx with trypsin, see the legend to Figure 4.

[^{55}Fe]Mesoheme was prepared by a procedure previously used (Davies et al., 1979). [^{55}Fe]Ferrous sulfate was from New England Nuclear. Mesoheme was dissolved in Me_2SO and its concentration measured in the same solvent by using a millimolar extinction coefficient of 170 at 393 nm. Because it is much more stable, mesoheme was used in preference to the naturally occurring heme. No difference in affinity of Hx for the two iron porphyrins in experiments in vitro or in vivo has been noted (Morgan & Muller-Eberhard, 1972). The reaction of apo-Hx incubated with trypsin was terminated by the addition of soybean trypsin inhibitor (STI; Millipore Corp., Freehold, NJ). [^{55}Fe]Mesoheme (36 000 cpm/sample) was added to the samples, which were incubated for 45 min at 37 °C. The incubation mixtures were applied onto 7% acrylamide gels and, after electrophoresis in this nondenaturing system, were analyzed by autoradiography.

Results and Discussion

The heme–agarose synthesized by Tsutsui and Mueller (1982a) has advantages over the matrices used in earlier methods of affinity chromatography [see references cited in Tsutsui and Mueller (1982a)] in not adsorbing albumin as well as in having a higher capacity for binding Hx. Chicken Hx, like mammalian Hxs, can be purified by a two-step procedure employing chromatography on heme–agarose and SP-Sephadex (Tsutsui & Mueller, 1982a,b). Because its net charge differs from that of the mammalian Hxs, chicken Hx is eluted from SP-Sephadex by 80–100 mM NaCl instead of the 180–200 mM NaCl required for the elution of Hxs of human, rat, and rabbit. As seen in Table I, recovery by this method was 36.5% for chicken Hx, nearly double the 19% yield for rat Hx. Recently Takahashi et al. (1985a) reported on a purification procedure for rabbit Hx employing heme–agarose affinity chromatography followed by ion-exchange HPLC.

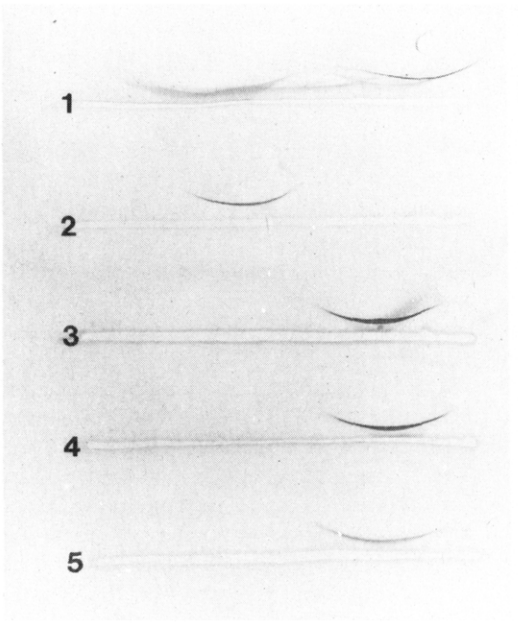

FIGURE 1: Comparison of the mobility of chicken, rat, rabbit, and human Hxs on immunoelectrophoresis. Antisera to the respective purified proteins were raised in rabbits against chicken Hx, or in goats against Hxs of rat, rabbit, and human, and rabbit anti-chicken serum was from Pel-Freez. Immunoelectrophoresis was performed on Gelbond films with 2% agar in phenobarbital buffer with 2% (w/v) PEG 4000, pH 8.2, for 3 h at 80 V and at room temperature. Antisera were applied after removal of the troughs, and the incubates were left overnight at room temperature in a moist chamber. The immunoprecipitation lines were stained with Coomassie blue and then destained in 40% methanol and 20% acetic acid. (1) Chicken serum in the well developed against anti-chicken serum in the trough; (2) purified chicken Hx developed against anti-chicken serum (upper trough, U) and anti-chicken Hx (lower trough, L); (3) rat Hx developed against anti-chicken Hx (U) and anti-rat Hx (L); (4) rabbit Hx developed against anti-rat Hx (U) and anti-rabbit Hx (L); and (5) human Hx developed against anti-rabbit Hx (U) and anti-human Hx (L). The anode is on the left and the cathode on the right.

The purified chicken Hx was tested for its heme and mesoheme binding capacity spectrophotometrically, and it was found to bind heme in a 1:1 molar ratio (data not shown). The circular dichroic spectrum of chicken Hx (data not shown) is very similar to that of rabbit and human Hx (Muller-Eberhard & Grizzuti, 1971; Morgan & Muller-Eberhard, 1972), which differ only minimally from each other in their amino acid and carbohydrate compositions (Hrkal & Muller-Eberhard, 1971; Takahashi et al., 1984) yet have distinct magnetic circular dichroic properties (Morgan & Vickery, 1978).

During assessment for purity on nondenaturing gradient PAGs, which showed a single band, it was observed that the Hx of chicken migrates faster than the Hx of all the other species (Figure 5). Consistent with this is the greater net negative charge indicated by the elution of the protein from SP-Sephadex at a lower ionic strength. On examination by immunoelectrophoresis, chicken Hx is indeed confirmed to be an α_1-globulin whereas the human, rat, and rabbit Hxs are β_1-globulins (Figure 1). Immunological cross-reactivities between the mammalian Hxs are demonstrated by the appearance of weak immunoprecipitation lines between rabbit Hx (well in 4, Figure 1) and anti-rat Hx (trough in 3), and between human Hx (well in 5) and anti-rabbit Hx (trough in 4); no cross-reactivity is discerned between rat Hx (well in 3)

Table II: Amino Acid and Carbohydrate Compositions of Chicken Hx and Rat Hx[a]

identity	chicken Hx[b]	rat Hx[c]	rabbit Hx	human Hx
Asx	43	41	45	39
Thr	17	18	24	23
Ser	24	39	32	30
Glx	52	37	38	37
Pro	43	33	29	35
Gly	53	40	42	43
Ala	40	35	26	28
Val	22	22	26	24
$^1/_2$-Cys	12	12	10	12
Met	5	4	4	6
Ile	12	12	11	9
Leu	39	35	37	37
Tyr	15	16	14	16
Phe	21	24	19	19
Lys	9	24	23	21
His	21	15	17	19
Arg	32	20	23	23
Trp	16	16	18	18
total	476	443	438	439
GlcNAc	14.4	16.4[d,e]	26.8	25.8[f]
Man	9.5	13.6	8.8	14.9
Gal	8.2	9.0	6.7	12.0
NeuNAc	7.7	13.8	18.6	16.7
GalNAc	0	0	0	1.0

[a] Compositional analysis of peptide chain of chicken Hx (based on a molecular mass of 52 kDa) and peptide chain of rat Hx (based on a molecular mass of 49 kDa). Amino acid values are given as nearest integral number of residues; average values are the nearest integral to the mean of the fractional numbers from each determination. Values for rabbit Hx and human Hx, provided for comparison, are from the amino acid analysis for rabbit Hx performed by Hrkal and Muller-Eberhard (1971) (based on a molecular mass of 49.35 kDa) and from the complete sequence determination of Takahashi et al. (1985b) for human Hx (giving an exact mass of 49.295 kDa). [b] Average of data from three determinations (R.B.T. and D.W.) with the exception of $^1/_2$-Cys and Trp, for which one determination only (R.B.T.) was done. [c] Average of data from two determinations (D.W.) except for $^1/_2$-Cys and Trp, for which one determination (R.B.T) was done. [d] Carbohydrate data of Bernard et al. (1975) adjusted to a molecular mass of 60 kDa. [e] This GlcNAc value (suspected low) is subject to conformation. [f] Carbohydrate data of Hrkal and Muller-Eberhard (1971) adjusted to (exact) molecular mass from sequence determination by Takahashi et al. (1985b).

and anti-chicken Hx (trough in 2). This previously observed finding, obtained by double diffusion in agar (Cox et al., 1978), had led us to believe either that the serum of avians has no Hx or that an avian Hx would be radically different in structure from mammalian Hxs. Immunoblotting experiments with polyclonal and monoclonal antibodies show, however, the existence of shared epitopes between the Hxs of mammals and avian Hx.

We also compared the electrofocusing pattern of chicken Hx with that of the other three Hxs, first in the pH range 3.5–9.5 and then in the pH range 4.0–5.0. Figure 2 demonstrates the molecular heterogeneity of chicken Hx on isoelectric focusing (IEF) in the pH range 4.0–5.0; at least nine bands are clearly visible. Each of the mammalian Hxs produces a broad smear of ill-defined bands in the same pH region. Similarly, chicken Hx shows a less complicated pattern on PAG electrophoresis under a number of conditions. On nondenaturing gels (5%, 7%, 9%, 10%, and 12% PAG; see Materials and Methods), chicken Hx behaves as a homogeneous species, unlike the other Hxs, which exhibit at least five to six bands (Figure 5). On SDS–PAG electrophoresis, chicken and rabbit Hx appear as a single band, yet the Hxs of both human and rat show several minor bands (not shown). For this reason we estimated the molecular weight of chicken Hx and rat Hx not only by SDS–PAG electrophoresis but also

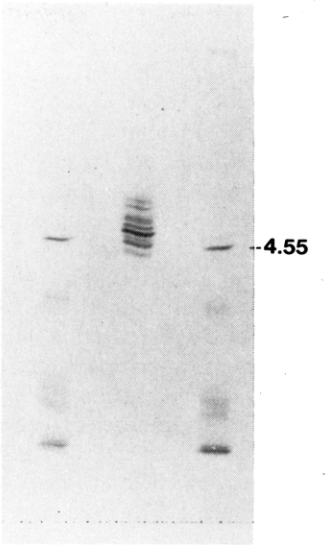

FIGURE 2: IEF electrophoresis of purified chicken Hx. Chicken Hx (50 μg in distilled water) was applied to ampholine PAG plates (pH 4.0–5.0). A mixture of protein standards (glucose oxidase, pH 4.15; soybean trypsin inhibitor, pH 4.55; β-lactoglobulin A, pH 5.20; bovine carbonic anhydrase B, pH 5.85; human carbonic anhydrase B, pH 6.55) was applied to wells on either side of the well containing chicken Hx.

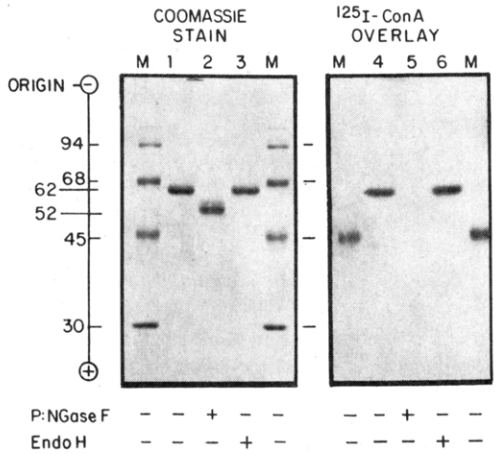

FIGURE 3: SDS–PAG electrophoresis of chicken Hx treated with P:NGase F and Endo H. Chicken Hx (10 μg) was denatured by heating in SDS and cooled, and excess SDS was complexed with Triton X-100. Samples were treated for 18 h at room temperature with P:NGase F (lane 2) or Endo H (lane 3). After electrophoresis, the gel was stained with Coomassie blue, photographed (lanes 1–3), and then overlayed with ^{125}I-ConA to label ConA-binding oligosaccharides. The gel was dried and autoradiographed on Kodak X-Omat AR film (lanes 4–6). The markers from Pharmacia were phosphorylase (94 kDa), bovine serum albumin (68 kDa), chicken ovalbumin (45 kDa), and carbonic anhydrase (30 kDa). Additional details are given under Materials and Methods.

by gel filtration. The latter method gave a molecular weight of chicken and rat Hx of 60 250. On SDS–PAG electrophoresis chicken Hx exhibits an apparent size of 62 ● 1.5 kDa (n = 8), and rat Hx, 71 ± 3 kDa (n = 4), the wider spread of values of the latter protein being caused by the appearance of several minor bands (see Figure 4A). The apparent molecular mass of both of these proteins is several kilodaltons less when the reducing agent is omitted, indicating that both proteins have a globular shape which fully unfolds only when the disulfide groups are reduced.

The compositional analysis of chicken Hx shown in Table II is based on a peptide size estimation of 52 kDa obtained when the protein was treated with P:NGase F to remove the carbohydrate moieties (lane 2 of Figure 3). The peptide

portion of rat Hx, synthesized by hepatocyte monolayers treated with the glycosylation inhibitor tunicamycin (Katz et al., 1985), is 49 kDa. The amino acid composition of rat Hx is very similar to the reported values for rabbit Hx (Hrkal & Muller-Eberhard, 1971), human Hx (Hrkal & Muller-Eberhard, 1971; Takahashi et al., 1985b), and sheep Hx (Stratil et al., 1984) whereas that of chicken Hx (Table II) is significantly different: the serine content and in particular the lysine content of chicken Hx are lower than for the other Hxs, and the content of glutamine/glutamate, arginine, proline, glycine, and histidine is higher than for the other Hxs. The avian protein does, however, retain the high tryptophan content characteristic of the mammalian Hxs. Given that the sum of the positively charged amino acids is approximately equal for all four species, the greater negative charge of chicken Hx appears to be due to its markedly higher Glx content (Table II), a significant proportion of which would be expected to be present as glutamic acid.

In addition to its substantially different amino acid profile, chicken Hx also displays differences in glycosylation compared with the human (Hrkal & Muller-Eberhard, 1971; Takahashi et al., 1984), rat (Bernard et al., 1975, 1983), and rabbit (Morgan & Smith, 1984) forms. The carbohydrate content of rat Hx has been provisionally calculated from the data of Bernard et al. (1975) adjusted for a protein mass of 49 kDa (Katz et al., 1985), which provides a holoprotein molecular mass of about 60 kDa. However, digestion of human and rat Hxs with P:NGase F followed by SDS–PAG electrophoresis reveals a comparable decrease in apparent size from about 72 to 50 kDa (data not shown), suggesting that the oligosaccharide component of rat Hx (in particular GlcNAc) has been underestimated.

The compositional analysis of chicken Hx (Table II) reveals the presence of GlcNAc, mannose, galactose, and sialic acid. Since no GalNAc was found, all oligosaccharides associated with chicken Hx should be N-linked through asparagine with none present O-linked to serine or threonine as in the case of human Hx (Takahashi et al., 1984). To characterize further the carbohydrate moieties, aliquots of chicken Hx, denatured with SDS, were digested either with P:NGase F, which hydrolyzes the glycosylamine bond of both complex and high-mannose N-linked glycans (Tarentino et al., 1985), or with Endo H, which hydrolyzes the di-*N*-acetylchitobiose core of high-mannose oligosaccharides (Tarentino et al., 1974; Trimble et al., 1978). Results of analysis of the treated samples by SDS–PAG electrophoresis are summarized in Figure 3. Lane 1 shows that the untreated chicken Hx migrates as a homogeneous species with an apparent size of 62 kDa. After treatment with P:NGase F, most of the chicken Hx migrates with an apparent size of about 52 kDa (lane 2), suggesting the release of approximately 10 kDa of carbohydrate. By contrast, treatment of denatured chicken Hx with Endo H (lane 3) fails to effect any change in migration, indicating the absence of associated high-mannose oligosaccharides.

The gel was overlaid with ^{125}I-ConA, which should bind to any biantennary complex glycans (Lis & Sharon, 1984). Autoradiography of the gel (Figure 3) reveals strong ConA binding onto both the untreated (lane 4) and Endo H treated (lane 6) Hx, while no label appears in the P:NGase F treated form (lane 5), indicating that all biantennary chains initially present are removed by the P:NGase F. The binding of ^{125}I-ConA by the ovalbumin marker (molecular mass of 45 kDa) serves as a positive experimental control. ConA does not bind efficiently to tri- or tetraantennary oligosaccharides (Lis & Sharon, 1984), precluding determination from the results in Figure 3 whether these species might also be present. Separate experiments (results not shown) with ^{125}I-WGA, which has affinity for both sialic acid and GlcNAc on all forms of complex oligosaccharides (Lis & Sharon, 1984), revealed weak binding to the 62-kDa form and to some species intermediate in size between 62 and 52 kDa (as seen in the Coomassie-stained band in Figure 3, lane 2). ^{125}I-WGA binding to these forms in the absence of ^{125}I-ConA binding suggests the presence of tri- or tetraantennary oligosaccharides.

Since there are no O-linked or N-linked high-mannose oligosaccharides, all carbohydrate present should be of the complex type with the invariant pentasaccharide core composed of a di-*N*-acetylchitobiose extended with 3 mannose residues (Kobata, 1984). On the basis of an estimate of 52 kDa (Figure 3, lane 2) for the carbohydrate-free peptide and the presence of 9.5 mannoses (Table II), there are 3.2 oligosaccharides/chicken Hx molecule. Subtracting the core-associated GlcNAcs [3.2 chains × $(GlcNAc)_2$/core = 6.4] from the value shown in Table II leaves a residue of 8 GlcNAc/Hx, which is the value obtained for the total galactose content (8.2) and sialic acid content (7.7) per Hx (Table II). Thus, the compositional analyses (Table II), the specificity of ConA and WGA binding, and the glycosidase digests all suggest that the three N-linked glycans of chicken Hx are a mixture of fully sialylated, fucose-free bi-, tri-, and possibly tetraantennary complex chains. Verification of this structure will have to await preparation of the protein in sufficient quantity for conclusive studies to be performed.

Rabbit Hx appears to have four biantennary chains (Morgan & Smith, 1984), while rat Hx, estimated from the 18% by weight carbohydrate, appears to have four or five N-linked glycans, about 70% of which are biantennary chains with the remainder being a mixture of triantennary structures and possibly some biantennary forms with an additional sialic acid (Bernard et al., 1983). The human form of Hx has five N-linked oligosaccharides and a single O-linked glycan blocking the N-terminal threonine residue (Takahashi et al., 1984). From compositional analyses[3] the human Hx oligosaccharide complement appears also to be a mixture of bi- and triantennary structures without fucose. Thus, chicken Hx appears to have the simplest carbohydrate makeup of all species studied to date.

To obtain additional details on the molecular characteristics of chicken Hx, we subjected it (in the presence and absence of heme) to proteolytic digestion with trypsin and plasmin and compared its sensitivity to proteolysis with that of the mammalian Hxs. The results of the digestion with trypsin are shown for iodinated Hxs in Figure 4. The presence of heme partially protects chicken Hx (lane 3 in Figure 4A), rat Hx (lane 6), and to a lesser extent rabbit Hx (lane 9) from digestion with trypsin. The uniqueness of the peptide map of each of the three Hxs is even more clearly recognized in the autoradiograph depicted in Figure 4B. The ^{125}I, which is probably linked mostly to tyrosine residues, associates only with a few peptides which are especially prominent in the case of rabbit Hx (lanes 8 and 9). Analysis of a similar experiment in which samples were applied to a 7% nondenaturing gel immediately after addition of an optimal amount of trypsin and after 5 min of incubation is depicted in Figure 5. This gel shows clearly the molecular heterogeneity for rabbit (lanes 5 and 6) and rat (lanes 13 and 14) of both the apoprotein and the mesoheme–Hx complex, respectively. Apo-Hx and mesoheme–Hx for chicken exhibit one or at most two bands (lanes

[3] R. B. Trimble, unpublished data; J. Baenziger, personal communication.

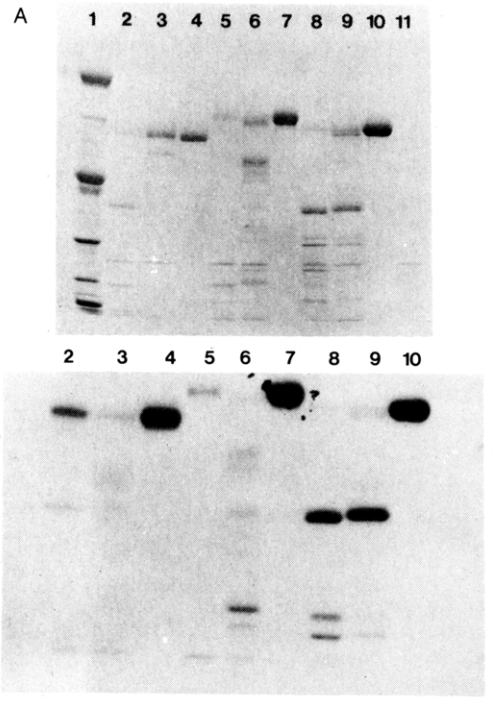

FIGURE 4: Gradient SDS–PAG electrophoresis of purified ^{125}I-labeled chicken, rat, and rabbit Hxs and partial proteolytic fragments after trypsin digestion in the presence or absence of heme. The ^{125}I-Hxs (15 μg of each Hx in 20 μL of 10 mM PBS, pH 7.4) were incubated with 0.34 μg of heme (1 μL in 0.1 M potassium phosphate buffer, pH 7.4) for 45 min at 37 °C or in buffer alone (apo-Hx). Samples were then incubated with 5 μg of trypsin (in 10 μL of 10 mM PBS, pH 7.4) or in buffer alone for 30 min at 37 °C. Incubation was terminated by adding an equal volume of solubilization buffer and boiling for 5 min. Samples (total volume) were loaded onto wells of a 7.5–15% gradient gel (SDS–PAG), essentially following the procedure of Laemmli (1970). The gel was fixed, stained, and destained as described under Materials and Methods. (A) Lane 1 [protein standards from Bio-Rad (5 μg of each)], phosphorylase B (92.5 kDa), BSA (66.2 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); lane 2, chicken apo-Hx + trypsin; lane 3, chicken Hx + heme + trypsin; lane 4, chicken Hx + heme; lane 5, rat apo-Hx + trypsin; lane 6, rat Hx + heme + trypsin; lane 7, rat Hx + heme; lane 8, rabbit apo-Hx + trypsin; lane 9, rabbit Hx + heme + trypsin; lane 10, rabbit Hx + heme; lane 11, trypsin. (B) Autoradiography of the above gel showing lanes 2–10 inclusive.

9 and 10). Again, all three iron porphyrin complexed Hxs incubated with trypsin (lanes 7, 11, and 15) prove more resistant to digestion than the apoproteins, in agreement with earlier results (Morgan & Smith, 1984; Takahashi et al., 1985a). The same gel pattern was obtained in similar experiments (not shown) with heme, which has a greater tendency than mesoheme to aggregate in aqueous media (Brown et al., 1970). Gels of plasmin-incubated Hxs of chicken, rat, and rabbit demonstrate (Figure 6) that chicken Hx (lane 2) and, in particular, rat Hx (lane 5) appear to be more resistant to plasmin digestion than rabbit Hx (lane 8), which resolves into two major bands as previously shown (Morgan & Smith, 1984). Heme protects all three Hxs from digestion by plasmin; this is best visualized for the Hx of rabbit (lane 9).

In conclusion, chicken Hx has been purified and studied, and certain of its properties have been compared with those of human, rat, and rabbit Hxs. Differing notably from the mammalian Hxs with respect to its electrophoretic mobility, the avian Hx proved by immunoelectrophoretic mobility to be an α_1-globulin. It has an amino acid composition distinct from the mammalian Hxs studied, all of which have similar profiles; and while the length of its peptide chain may be slightly longer, it exhibits a simpler carbohydrate structure

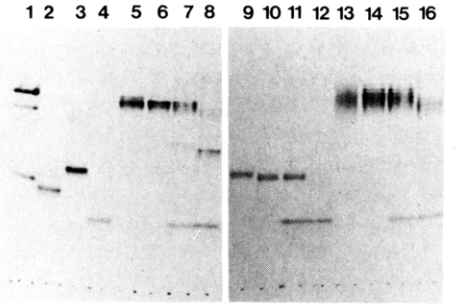

FIGURE 5: Influence of heme on electrophoretic patterns in nondenaturing gels of purified chicken, rat, and rabbit Hxs and partial proteolytic fragments after trypsin digestion. Hxs (15 μg in 15 μL of 10 mM PBS, pH 7.4) were incubated with 5 μg of trypsin (in 10 μL of 10 mM PBS, pH 7.4) for 0 or 5 min at 37 °C, or for 5 min in buffer alone. Reactions were terminated by addition of soybean trypsin inhibitor (15 μg in 5 μL of 10 mM PBS, pH 7.4). Samples were incubated with 0.25 μg of mesoheme (in 4 μL of Me_2SO) for 45 min at 37 °C or in Me_2SO alone. Samples were mixed with equal volumes of sample buffer and applied to a 7% nondenaturing gel. Lanes 1–3, protein standards from Sigma [lane 1, carbonic anhydrase (20 μg; 29 kDa) and α-lactalbumin (15 μg; 14.2 kDa); lane 2, chicken ovalbumin (20 μg; 45 kDa); lane 3, BSA [15 μg; 132 kDa (dimer) and 66 kDa (monomer)]]; lane 4, trypsin (upper band) and STI (lower band); lane 5, rabbit apo-Hx; lane 6, rabbit Hx + heme; lane 7, rabbit Hx + heme + trypsin + STI (0 min); lane 8, rabbit Hx + heme + trypsin (5 min) + STI; lane 9, chicken apo-Hx; lane 10, chicken Hx + heme; lane 11, chicken Hx + heme + trypsin + STI (0 min); lane 12, chicken Hx + heme + trypsin (5 min) + STI; lane 13, rat apo-Hx; lane 14, rat Hx + heme; lane 15, rat Hx + heme + trypsin + STI (0 min); lane 16, rat Hx + heme + trypsin (5 min) + STI.

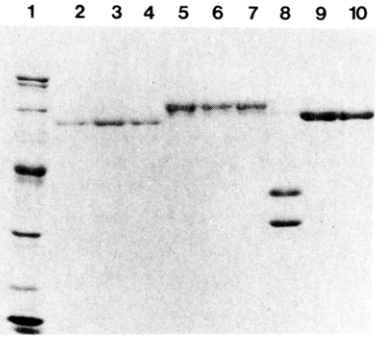

FIGURE 6: Gradient SDS–PAG electrophoresis of purified Hxs of chicken, rat, and rabbit Hxs and partial proteolytic fragments after plasmin digestion. Hxs (approximately 15 μL of each in 10 mM PBS, pH 7.4) were incubated with 0.25 ng of heme (4.2 μL in 0.1 M potassium phosphate buffer, pH 7.4) for 45 min at 37 °C, or in buffer alone. Samples were then incubated with 0.025 unit of plasmin (2.5 μL in 10 mM PBS, pH 7.4) for 2 h at room temperature, or in buffer alone. Reactions were terminated by addition of an equal volume of solubilization buffer and by boiling for 5 min. Samples (total volume) were loaded onto wells of a 7.5–15% gradient gel (SDS–PAG), and the gel was fixed, stained, and destained as described under Materials and Methods. Lane 1, protein standards (as in Figure 4) and plasmin, which at the concentration used was not visualized; lane 2, chicken apo-Hx + plasmin; lane 3, chicken Hx + heme + plasmin; lane 4, chicken Hx + heme; lane 5, rat apo-Hx + plasmin; lane 6, rat Hx + heme plasmin; lane 7, rat Hx + heme; lane 8, rabbit apo-Hx + plasmin; lane 9, rabbit Hx + heme + plasmin; lane 10, rabbit Hx + heme.

than these other forms. Like the mammalian Hxs, chicken Hx binds heme and mesoheme in an equimolar ratio, and this heme binding confers resistance to proteolytic digestion.

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