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Identification of apolipoprotein A-I in the α -globulin fraction of avian plasma

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Key Words

Agarose gel electrophoresis, α -globulin, apolipoprotein A-I, avian, plasma protein electrophoresis

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Background: Plasma protein electrophoresis is frequently used in birds as a tool for the diagnosis and monitoring of disease. Identification of proteins in individual peaks can help improve our understanding of changes in protein concentration in physiologic and pathologic conditions.

Objective: The aim of this study was to verify the presence and identify the protein(s) in the prominent α -globulin peak of orange-winged parrots (*Amazonica*), black kites (*Milvus migrans*), and rock pigeons (*Columba livia*).

Methods: Heparinized plasma samples were obtained from 12 birds of each species. Agarose gel electrophoresis and total protein concentration were determined using standard techniques. One plasma sample from each species was then electrophoresed using high-resolution agarose gels to isolate the α -globulin band. Gel strips were digested in trypsin and peptides were extracted and analyzed using liquid chromatography with tandem mass spectrometry. De novo sequencing was used to identify the protein based on homology scoring against a protein database.

Results: Electrophoresis verified the presence of a single prominent α -globulin peak, usually in the α_1 -region, that had a median concentration of 9.4 g/L (range, 2.1–11.7 g/L, 21.6% of total protein) in parrots, 12.2 g/L (10.4–13.2 g/L, 35.9%) in kites, and 10.7 g/L (9.0–11.5 g/L, 40.0%) in pigeons. Mass spectrometry and sequencing analysis unequivocally identified the protein as a mature circulating form of apolipoprotein A-I (apo A-I) in all 3 species.

Conclusions: Apo A-I accounts for the prominent α -globulin peak and comprises a major proportion of total protein concentration in diverse avian species. As a high-density lipoprotein and negative acute phase protein with a pivotal role in cholesterol homeostasis, further study is warranted to determine the significance of changes in apo A-I concentration in avian electrophoretograms.

Introduction

Within the last 15 years, the application of protein electrophoresis in clinical avian medicine has received considerable attention, and it is recognized as a useful diagnostic tool for the evaluation, diagnosis, and monitoring of a variety of diseases and conditions.^{1–3} Plasma proteins in electrophoretic gels are identified according to their electrophoretic mobility and immunoreactivity as either albumin or α -, β -, or

γ -globulins.³ Some authors have demonstrated high intertaxonomic variations in plasma electrophoresis patterns.^{2,4,5}

We regularly use plasma protein electrophoresis as a diagnostic tool in our laboratory and have observed a prominent peak in the α -globulin region in electrophoretic tracings from birds in various taxa including Columbiformes, Falconiformes, Strigiformes, Psittaciformes, Phoenicopteriformes, Charadriiformes, and Ciconiiformes (Y.R., unpublished data). Although

the amplitude of the peak seems to depend on the species, its size is sufficiently large in some species such that it could lead to errors in the interpretation of electrophoretograms. Although 2 recent studies have noted the existence of this peak, neither has identified its origin.^{6,7} The purpose of the present study was to verify the presence and determine the composition of this unique α -globulin peak in agarose gel electrophoretograms from 3 phylogenetically distant bird species.

Materials and Methods

Experimental animals and samples

The study was conducted on 12 rock pigeons, *Columba livia* (5 males, 7 females) at the zoological park of Clères (France), 12 black kites, *Milvus migrans* (7 males, 5 females) at the Académie de fauconnerie du Puy du fou (France), and 12 orange-winged parrots (*Amazona amazonica*) of unknown sex at the Zoo de Lille. Blood samples were performed on the occasion of a veterinary screening protocol. All of the birds were examined and determined to be clinically healthy.

Blood samples were taken from the right jugular vein of the black kites and orange-winged parrots, which is the preferred sampling site in avian species whose jugular veins are overlaid by a featherless tract of skin (apterium), and from the brachial vein of the rock pigeons. Samples were taken outside the breeding season, from September to December 2007, depending on the species, using 23 G needles and 2 mL syringes (Terumo Europe N.V., Leuven, Belgium). Two milliliters of blood were drawn from each bird into lithium heparin Venosafe-evacuated blood collection tubes (Terumo Europe N.V., Leuven, Belgium), and centrifuged at 3000g for 5 minutes. Plasma was removed and stored in cryotubes (Micronic Systems, Lelystad, The Netherlands) at -20°C for 1–3 weeks until analysis.

Total protein concentration and plasma protein electrophoresis

Samples were thawed and rehomogenized by gentle mixing 1 hour before analysis. Total protein concentration was determined by the biuret reaction at 552 nm, using a Roche Integra 400 chemistry analyzer (Roche Diagnostics, Meylan, France). Agarose gel electrophoresis of plasma proteins was done using a Hydrasys semi-automated system (Sebia, Evry, France) and a Hydragel protein 15/30 set (Sebia). The system was operated according to the manufacturer's instructions, using version 7.00 F0.1 of the system software. Plasma samples (10 μL) were applied manually and

were allowed to diffuse for 5 minutes in a wet chamber. Sample application (30 seconds), electrophoresis (~ 7 minutes), and drying (65°C for 10 minutes) were done automatically in the migration compartment of the instrument. The temperature was maintained at 20°C using a Peltier device. Electrophoretic separation was obtained on 8 g/L agarose gels in a Tris-barbital buffer (pH 9.2), at constant power level of 20 W, until 33 V h had been accumulated. Dried gels were transferred manually to the staining compartment and stained automatically for 4 minutes with 4 g/L amidoblack in an acidic solution, destained (3 times, for 3 and 2 minutes, and 1 minute) with 0.5 g/L citric acid solution, and dried at 75°C for 8 minutes. Gels were then scanned with a high-resolution Epson Perfect V700 photo scanner (Epson France, Nanterre, France).

Electrophoretic curves and percentage values of the different fractions were determined using Phoresis software (version 5.50, Sebia). Albumin was identified as being the strongest anodal peak. In all of the species, the α fraction of interest was located in a position just cathodic to that of albumin. The concentration of protein in the α -globulin fraction was determined by multiplying the relative fraction percentage by the total protein concentration.

Separation and digestion of the α -globulin band

One plasma sample from each species was chosen randomly for electrophoresis using Hydragel 15HR high-resolution gels (Sebia). Each sample was run twice on 2 distinct high-resolution gels. The first one was dried and stained according to the manufacturer's instruction and was used to locate the α band. The second one, for which the semi-automatic process was interrupted before the drying and staining steps, was used to cut out the α band of interest. The latter was accurately located by superimposing the nondried gel with the stained one. Gel strips cut out from the gel were placed in Eppendorf tubes (Eppendorf GA, Hamburg, Germany), and mailed under refrigerated conditions (3°C) to the PAPSS (Plateau d'Analyse Protéomique par Séquençage et Spectrométrie de Masse) proteomic platform at INRA (Institut National de Recherche Agronomique, Jouy en Josas, France), for peptide de novo sequencing and protein identification.

Each gel strip was washed twice with 200 μL 50 mM ammonium carbonate in a 50% acetonitrile solution, dried at room temperature, and digested overnight at 37°C with 100 ng of sequencing-grade modified trypsin (Promega, Madison, WI, USA) in 2 μL of 50 mM NH_4HCO_3 . The resulting peptides were extracted as follows: the supernatant from trypsin

hydrolysis was first transferred into a new tube, and the gel slices were extracted once with 25 μ L of buffer extract-B (50 mM ammonium carbonate), and twice with 25 μ L of buffer extract-C (formic acid 0.1% acetonitrile 50%). For each extraction, the gel slices were incubated for 15 minutes at room temperature under gentle shaking conditions. The 3 extracts were pooled with supernatant from the original trypsin digest and dried for 1 hour in a Speed-Vacuum concentrator (Savan, Thermo Fisher, Illkirch, France). The peptides were then resuspended in 25 μ L of precolumn loading buffer (0.08% trifluoroacetic acid and 2% acetonitrile in water), before analysis.

Mass spectrometric analysis

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis was performed with an Ultimate 3000 LC system (Dionex, Voisins le Bretonneux, France) connected by a nanoelectrospray interface to a linear ion trap mass spectrometer (LTQ, Thermo Fisher, Waltham, MA, USA). Four microliters of tryptic peptide mixtures from each sample were loaded at a flow rate of 20 μ L/min onto a precolumn (Pepmap C18, 0.3 \times 5 mm, 100 \AA , 5 μ m; Dionex). After 4 minutes, the precolumn was connected to a separating Pepmap C18 nanocolumn (0.075 \times 15 cm, 100 \AA , 3 μ m; Dionex) and the gradient was set to 300 nL/min. All peptides were separated in the nanocolumn using modified buffer elut-B with a linear gradient of acetonitrile from 2% to 36%, for 18 minutes. The eluting buffers were buffer elut-A: 0.1% formic acid, 2% acetonitrile and buffer elut-B: 0.1% formic acid, 80% acetonitrile. The total run length was 50 minutes, including the regeneration step. Ionization was performed at the liquid junction, with a spray voltage of 1.3 kV applied to a noncoated capillary probe (PicoTip Emitter, 10 μ m ID; New Objective, Woburn, MA, USA). The peptide ions were analyzed using the Nth-dependent method as follows: (1) full mass spectrometry scan (m/z 300–2000); (2) ZoomScan (scan of the 3 major ions); and (3) MS/MS on these 3 ions using standard peptide fragmentation parameters ($Q_z=0.25$, activation time = 30 ms, collision energy = 40%).

Protein identification by de novo sequencing and homology scoring

Protein identification was carried out using Peaks Studio 4.2 software (Bioinformatic Solution, Waterloo, ON, Canada), together with the FASTS tools (available for free at: <http://www.ebi.ac.uk/fasta33/>). Raw data were first loaded into Peaks Studio and filtered to eliminate noisy spectra. Filtered MS/MS spectra were rap-

idly translated into amino acid sequences, with several de novo sequencing parameters: parent and fragment-mass error tolerances of 0.5 Da, trypsin as the protease, with a maximum of 1 missed cleavage allowed, and partial oxidation of methionine. All of the sequences produced by Peaks Studio were then filtered, so as to retain only those with peaks scoring higher than 50% on doubly charged ions. These short sequences were analyzed with FASTS against the Uniref100 protein database, with MDM20 as the selected matrix. The identified proteins were classified by homology scoring, according to their expectation value (E -value), reflecting the significance of the score.

Statistical analysis

Descriptive statistics were calculated (Microsoft Excel 2003, Microsoft Corp., Redmond, WA, USA). Kruskal–Wallis 1-way ANOVA was used to study the difference in α -globulin fractions concentrations between species and Mann–Whitney U -test was used to determine differences in α -globulin fractions concentrations between sexes within each species (Systat 7.0, Systat Software Inc., London, UK).

Results

The electrophoretic patterns of the 3 species were similar and included a prominent narrow peak in the α -globulin fraction (Figure 1). However, the studied species differed from one another in their α -globulin concentration ($U=21.275$; $P<.01$). The α -globulin peak had a median concentration of 9.4 g/L (range, 2.1–11.7 g/L; 21.6% of total protein) in parrots, 12.2 g/L (10.4–13.2 g/L, 35.9%) in kites, and 10.7 g/L (9.0–11.5 g/L, 40.0%) in pigeons. No significant difference was found between males and females in the α -globulin fractions within the 3 species studied.

LC-MS/MS analysis of peptides resulting from trypsin digestion of proteins in the α -globulin peak is shown in Figure 2. Based on homology with sequences in the Uniprot database, apolipoprotein A-I (apo A-I) was identified as the main protein in the α -globulin peak in samples from all 3 species. The highest similarity was observed with apo A-I from duck, chicken, quail, and turkey, in which highly significant hits (E -values, 1.9×10^{-34} – 6.5×10^{-71}) with large coverage (49.6–74.8% of hit sequence length) corresponded to apo A-I. The 24 amino acids of the apo A-I amino-terminal region were not represented in fingerprints, probably indicating a truncated mature form of the protein. Few less-significant hits corresponded to more distantly related mammalian apo A-I. All other

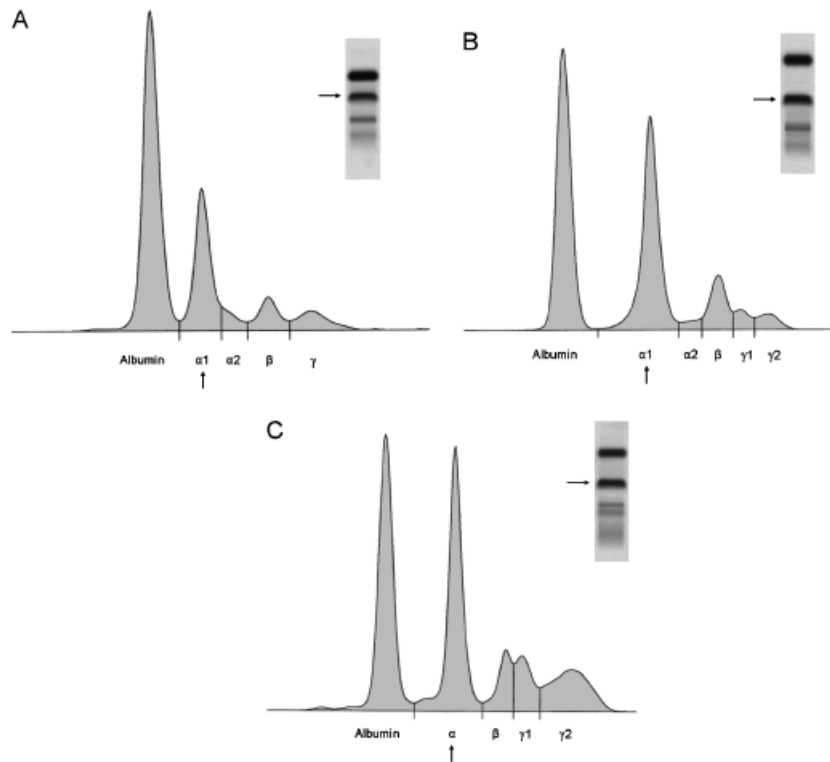


Figure 1. Representative plasma protein electrophoretic pattern from an (A) orange-winged parrot (*Amazona amazonica*), (B) black kite (*Milvus migrans*), and (C) rock pigeon (*Columba livia*). The α -globulin peak of interest is indicated with an arrow.

significant hits corresponded to human keratins and cytokeratins, which were interpreted as contaminants, and to porcine trypsin, which was used during sample preparation to generate peptides.

Discussion

For the first time, a high-amplitude peak in the α -globulin region of birds was identified, as apo A-I. The same apolipoprotein peak was observed in 3 species belonging to distant bird taxa (Psittaciformes, Falconiformes, and Columbiformes).

We found only 2 articles written in the past 30 years that mention the existence of a prominent peak in the α -globulin fraction similar to that described in the present study. Tatum et al.⁸ described a high-amplitude α_1 -globulin fraction in Falconiformes and Strigiformes that was positioned just at the edge of the albumin peak. More recently, pigeons were found to have relatively high α -globulin concentrations, in comparison with ducks and turkeys.⁹ Neither of these studies shed light on the molecular origin of the protein(s) in the α -globulin peak. It is interesting to note

that these studies are also the only ones that used agarose gel electrophoresis for evaluating plasma proteins from Falconiformes and Columbiformes; all other studies used cellulose acetate electrophoresis (Falconiformes^{10–13} and Columbiformes^{14,15}). The poorer resolution of the latter technique, in comparison with agarose, could be the reason why the α -globulin peak was not observed. To our knowledge, a prominent peak in the α -globulin region has not been described previously in Psittaciformes in studies based on agarose gel⁶ and cellulose acetate^{16–18} electrophoresis. As described earlier in Falconiformes and Columbiformes, this could be related to the poorer resolution of cellulose acetate electrophoresis. However, in their recent article on plasma protein electrophoresis in Psittacidae, Cray et al.⁶ obtained reference values for α -globulin fractions in multiple species that did not exceed 9% of the total protein content with agarose gel electrophoresis. This could be because of the fact that their electrophoretic parameters (Beckman Paragon SPEP-II gel system; migration obtained with 100 V for 37 minutes) were different from those used in our study.

The comparison of peptide sequences obtained from the α -globulin bands with protein sequences in

		10	20	30	40	50	60
Human	P02647	MKAAVLT	LAVLFL	TGSGQARHFWQ	DEPPQSPFWD	RVKDLATV	VYDVLKDSGRDYVSQFEGS
Chicken	P08250	MRGVLVTL	LAVLFL	TGTQARSFQ	HDEP-QTPLDR	IRDMVDVY	LETVKASGKDAIAQFESS
Quail	P32918	MRGVLVTL	LAVLFL	TGTQARSFQ	HDDP-QTPLDR	IRDMLDVY	LETVKASGKDAISQFESS
Duck	042296	MRVVVVTL	LALLFL	TGTQARYFWQ	HDEP-QAPLDR	LDLVDVY	LETVKASGKDAIAQFEAS
Pigeon					DEP-KAPLDR	DMVDVY	LESLK WLAQFESS
Kite					MLA-QTPLDR		WLAKFESS
Parrot					DEP-QAPLDR		WLAKFESS
		70	80	90	100	110	120
Human	P02647	ALGKQLNL	KLDNWD	SVTSTF	SKLREQL	GPVTQEF	WDNLEKETEGLRQEMSKDLEEVKAK
Chicken	P08250	AVGKQLDL	KLADNLD	TL SAAAAK	REDMAP	YYKEVRE	MWLKDTALRAELTKDLEEVKEK
Quail	P32918	AVGKQLDL	KLADNLD	TL SAAAAK	REDMT	PYYEVRE	MWLKDTALRAELTKDLEEVKEK
Duck	042296	AVGKQLDL	KLADNLD	TL GAAAAK	REDMAP	YYKEVRE	MWLKDTESLRAELTKDLEEVKEK
Pigeon		AVGK	LADNLD	TL SAAVAK	REDVTP	PYYK	EFWLK
Kite		AVGK	LAENLDM	L GAAAAK	LEDMT	PYYK	EFWLK
Parrot		AVGK	LTDNLD	TL GAAAAK	REDFAP	PYYK	EFWLK
		130	140	150	160	170	180
Human	P02647	VQPYLDDF	QKKWQEM	ELYRQK	VEPLRAEL	QEGARQ	KLHELQEKLSPLGEEHRDRARAHV
Chicken	P08250	IRPFLLDQ	SAKWTEE	EQYRQRL	TPVAQEL	KELTKQ	KVELMQAKLTPVAEEARDRLRGHV
Quail	P32918	IRPFLLDQ	SAKWTEE	EQYRQRL	LAPVAQEL	KDLTKQ	KVELMQAKLTPVAEEVRDLREQV
Duck	042296	IRPFLLDQ	SAKWTEE	EQYRQRL	LAPVAEEL	KELTKQ	KVELMQAKLTPVAEEARDRLRGHV
Pigeon		LASYLTK	WTEELEK	YR LVLAKEL	K QQVEL	VKERL	TPVAEEAR GFE
Kite		LRPFNNEF	SAKWTEE	LEKYLAPVAQEL	K APAVEL	MQEKL	TPVAEDVR RLEHV
Parrot		LASYLTK	WTEELEQ	YR LAPVAKEL	K		LTPVAEEAR
		190	200	210	220	230	240
Human	P02647	DALRTHL	LAPYSDEL	RQRLAAR	LEALKEN	GARGAR	LAEYHAKATEHLSTLSEKAKPALEDLRQ
Chicken	P08250	EELRKNL	LAPYSDEL	RQKLSQ	KLEEIRE	KGIPQ	ASEYQAKVHEQLSNLREKMTPLVQEFRE
Quail	P32918	EELRKNL	LAPYSSEL	RQKLSQ	KLEEIRE	KGIPQ	ASEYQAKVVEQLSNLREKMTPLVQEFKE
Duck	042296	EELRKNL	LAPYSDEL	RQKLSQ	KLEEIRE	KGIPQ	AAEYQAKVVEQLSNLREKMTPLVQDFKE
Pigeon		EELR	NLANESDEL				
Kite		EELR	NLAPYSNELR		GLPKATEY	KAKVVEHLSNLR	FTPLLKEFK
Parrot			NLAPYSDEL			VVEHLSNLR	FTPLEKEFK
		250	260				
Human	P02647	GLLPVLES	FKVSFL	SALEEY	TKKLN	TQ	
Chicken	P08250	RLTPYAEN	LKNRLI	SFLDEL	QK	SVA	
Quail	P32918	RLTPYAEN	LKNRLI	IDLDEV	QK	THA	
Duck	042296	RLTPYAEN	LKTRFI	SLLDEL	QK	TVA	
Pigeon		ALPYAESL	K LLALL	DDFK			
Kite		TLPYAENL	K FLTLL	DDLK			
Parrot		ATPYAENF	K MEAFL	DDLK			

Figure 2. Sequence alignment of proapolipoprotein A-I from human (*Homo sapiens*, UniProt P02647), chicken (*Gallus gallus*, UniProt P08250), quail (*Coturnix japonica*, UniProt P32918), duck (*Anas platyrhynchos*, UniProt O42296), and turkey (*Meleagris gallopavo*, UniProt A4GUJ5) with peptides resulting from trypsin digestion of the protein contained in the α -globulin band in plasma from a black kite (*Milvus migrans*), a rock pigeon (*Columba livia*), and an orange-winged parrot (*Amazona amazonica*). The peptide sequences correspond to those determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and de novo sequencing.

the Uniprot database allowed us to unequivocally identify apo A-I as the main or only protein in the peak. The absence of the expected 24 N-terminal amino acids, based on alignment with the chicken sequence, could mean that the apolipoprotein in the 3 species we studied was a mature circulating form. The missing sequence corresponds to an 18-amino-acid prepeptide, which may act as a signal peptide, and to a 6-amino-acid propeptide.^{7,19,20} Indeed, the conversion of proapo A-I to mature apo A-I requires the proteolytic cleavage of these 24 amino acids.⁷ Some small sequences were not identified in this study. This is likely because trypsin systematically cuts peptides at the C-terminal of lysine or arginine. If they are too

small, such peptides cannot be identified by mass spectrometry.

Apo A-I is the most abundant apolipoprotein in the high-density lipoprotein (HDL) fraction in both birds and mammals.^{7,21,22} Apo A-I is an amphipathic protein, in which hydrophilic regions interact with polar surface lipids and hydrophobic regions interact with core lipids. These interactions contribute to the stability of apo A-I and allow the solubilization of hydrophobic lipids in an aqueous environment such as blood.²³ In birds, HDLs are responsible for the redistribution of cholesterol, cholesterol esters, and other nonpolar lipids from peripheral tissues to the liver for reuse or excretion in the bile.^{23,24} Apo A-I therefore

plays a pivotal role in cholesterol homeostasis, through reverse cholesterol transport.^{23,25}

In the present study, apo A-I appeared to be very abundant in pigeons and black kites, where the α -globulin peaks comprised up to one-third of the total protein content. This was consistent with findings in other studies indicating that HDL and cholesterol concentrations in pigeons are higher than those in most other avian species, with total plasma cholesterol concentration averaging 7.7 mmol/L, and 75–80% of cholesterol present in the form of HDL.^{26,27}

High concentrations of apo A-I could be the result of an adaptation to the peculiar lipid metabolism of certain types of birds in which fatty acids are the primary source of fuel during long-distance flights.^{28,29} This could explain the lower concentration of apo A-I in orange-winged parrots (a sedentary species) compared with black kites and pigeons, which are migrant birds. High apo A-I concentration could also be the result of adaptation to potentially high cell membrane turnover associated with the higher metabolic rate of birds compared with mammals of the same weight.^{30–32} Further studies are needed to investigate apo A-I peaks in other avian species over a range of physiologic conditions such as egg laying, molting, premigratory hyperphagia, and migration. Such investigations may lead to a more thorough understanding of the role of apo A-I in birds.

The high concentration of apo A-I and its presence in the α -globulin region of the 3 species studied must be taken into account when interpreting electrophoretograms. Such a peak could be incorrectly interpreted as resulting from inflammation, because numerous positive acute phase proteins such as α -1 antitrypsin and α -2 macroglobulin have been reported to migrate into the α -region in birds.² Apo A-I, like albumin, is a negative acute phase protein in chickens³³ (as well as in humans^{34,35}) such that its plasma concentration decreases in inflammation and may affect the albumin:globulin ratio.

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