Characterization of poultry egg-white avidins and their potential as a tool in pretargeting cancer treatment

Vesa P. HYTÖNEN*, Olli H. LAITINEN*¹, Alessandro GRAPPUTO*, Anu KETTUNEN*, Janne SAVOLAINEN†, Nisse KALKKINEN‡, Ari T. MARTTILA*², Henri R. NORDLUND*, Thomas K. M. NYHOLM§, Giovanni PAGANELLI|| and Markku S. KULOMAA*³

*Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box 35 (YAB), Jyväskylä, FIN-40014, Finland, †Department of Chemistry, University of Jyväskylä, P.O. Box 35, Jyväskylä, FIN-40014, Finland, ‡Institute of Biotechnology, University of Helsinki, P.O. Box 65, Helsinki, FIN-00014, Finland, §Department of Biochemistry and Pharmacy, Åbo Akademi University, P.O. Box 66, Turku, FIN-20521, Finland, and ||Division of Nuclear Medicine, European Institute of Oncology, Milan, Italy

Chicken avidin and bacterial streptavidin are proteins used in a wide variety of applications in the life sciences due to their strong affinity for biotin. A new and promising use for them is in medical pretargeting cancer treatments. However, their pharmacokinetics and immunological properties are not always optimal, thereby limiting their use in these applications. To search for potentially beneficial new candidates, we screened egg white from four different poultry species for avidin. Avidin proteins, isolated from the duck, goose, ostrich and turkey, showed a similar tetrameric structure, similar glycosylation and stability against both temperature and proteolytic activity of proteinase K as chicken avidin. Biotin-binding properties of these avidins,

measured using IAsys optical biosensor, were similar to those found in avidin from the chicken. Three of these novel avidins, however, showed different immunological cross-reactivities when compared with chicken avidin. The patient sera responses to duck, goose and ostrich avidins were also lower than those observed for chicken and turkey avidins. Our findings suggest that the use of these proteins offers advantages over chicken avidin and bacterial streptavidin in pretargeting applications.

Key words: avidin–biotin technology, biotin-binding protein, cancer treatment, immunoreactivity, targeted drug delivery.

INTRODUCTION

Chicken avidin and bacterial streptavidin are tetrameric proteins, which share an exceptionally high level of affinity with the water-soluble vitamin biotin ($K_d \approx 10^{-15}$ M) [1]. This tight interaction has made it possible to use (strept)avidin and biotin as probes and affinity tags in a wide variety of applications in the life sciences [2]. These new (strept)avidin–biotin techniques are also found to be useful in medical applications *in vivo* to localize and image cancer cells and to pretarget drugs to tumours [3–6]. In addition, the utilization of avidin in drug delivery through the blood–brain barrier has been demonstrated previously [7].

In spite of the fact that avidin and streptavidin are structurally and functionally analogous proteins, they differ in their primary amino acid sequence, pI, glycosylation [8] and immunological reactivity [9]. Each avidin monomer has one N-linked carbohydrate side chain, whereas streptavidin is devoid of sugars. The pI of avidin is very high (approx. 10.5), whereas streptavidin has a slightly acidic pI (approx. 6) [8]. Owing to these dissimilarities, the pharmacokinetics of avidin and streptavidin are rather different [10–12]. The plasma half-life of avidin [10] is shorter when compared with streptavidin [13]. Both the high pI and the glycosylation of avidin have been suggested to be responsible for the fast clearance of avidin. Owing to glycosylation, avidin has a tendency to accumulate in the liver, whereas its accumulation in the kidneys is mainly caused by its high pI [14]. Streptavidin, on the other hand, accumulates mainly in the kidneys [10].

Pretargeting strategies in cancer therapy rely on the use of monoclonal antibodies (mAbs), which recognize tumour-specific antigens in cancer cells. Avidin and streptavidin have found uses in multi-step pretargeting trials because of their extraordinarily tight biotin binding and also because, as tetramers, they provide signal amplification, leading to the accumulation of higher effective doses of radionuclides or other biotinylated drugs when compared with other immunotherapeutic procedures [15–17]. In pretargeting applications, avidin has been used as a fusion protein with an antibody [18], as a linker between biotinylated antibody and biotinylated radionuclide [19], as well as a carrier to remove free biotinylated antibodies [20].

A three-step approach in pretargeting has been designed to improve the target/non-target ratio, and clinical trials have already been performed in glioma patients [6,21,22]. Biotinylated mAbs are first injected (step 1), followed by injection of avidin and streptavidin 1 day later (step 2) [21]. The second injection has a 2-fold purpose: (i) removal of the excess circulating biotinylated antibodies in the form of unlabelled complexes via avidin (fast clearance), and (ii) targeting of tumour cells with streptavidin (slower clearance). Thereafter, radiolabelled biotin, which will selectively bind to 'avidinylated' tumour, is injected (step 3). The three-step approach is designed to remove the excess circulating biotinylated mAbs as unlabelled complexes, which are taken up and metabolized in the liver. This is the major factor in background reduction, and is obtained before injecting radiolabelled biotin. Moreover, the rapid blood clearance of the radiolabelled biotin allows imaging

Abbreviations used: AP, alkaline phosphatase; DSC, differential scanning calorimetry; endo H_r, recombinant endoglycosidase H; mAb, monoclonal antibody.

¹ Present address: Department of Molecular Medicine, A. I. Virtanen Institute, University of Kuopio, P.O. Box 1627, Kuopio, FIN-70211, Finland.

² Present address: Department of Molecular Medicine, National Institute of Health, Biomedicum, P.O. Box 104, Helsinki, FIN-00251, Finland.

³ To whom correspondence should be addressed (e-mail markku.kulomaa@csc.fi).

to be performed only 90–120 min after injection with low background activity. In addition, signal amplification may occur if tetrameric avidin binds more than one molecule of radioactive biotin. However, avidin and streptavidin are immunogens, which may stimulate immunological response against the proteins, preventing their repeated use [17,23,24]. Moreover, the pharmacokinetic properties of these native proteins are not quite optimal [10,11].

To overcome these immunological problems and to improve their pharmacokinetics, chemically modified forms of avidin and streptavidin have been developed. Rosebrough and Hartley [12] deglycosylated and chemically lowered the pI of avidin, and attached galactose to streptavidin to modify their pharmacokinetics. Subsequently, Chinol et al. [25] succeeded in reducing the immunological response and increasing the plasma half-life of avidin by poly(ethylene glycol)ylation and succinylation. Recently, Meyer et al. [26] constructed a streptavidin mutant with decreased antigenicity in rabbits. Several other recent reports have also described genetically modified avidins and streptavidins, which should offer advantages over wild-type proteins [26–32]. However, each solution has so far had its own drawbacks, which means that the need for a better understanding of the immunological properties of the various components of the treatment, as well as the need for immunologically and pharmacokinetically improved proteins, continues to exist.

It appears that one important source has been under-rated in the quest for better forms of avidin for applications *in vivo*: Mother Nature. The expression of avidin has been documented in avian, amphibian and reptilian egg-laying species [33–35]. It is, however, in only a limited number of studies that these non-chicken avidins have been described in any detail. All the application-related studies and improvements have concentrated on chicken avidin and bacterial streptavidin and their modified or mutated forms. In this context, we report in the present study on the isolation and characterization of avidins from four birds, the turkey (*Meleagris gallopavo*), domestic duck (*Anas platyrhynchos*), domestic goose (*Anser anser*) and ostrich (*Struthio camelus*). The main objective was to study whether they could offer advantages over natural and modified chicken avidins in pretargeting applications. We also explored the diversity of these avidin forms from the physicochemical and evolutionary points of view.

EXPERIMENTAL

Protein purification, N-terminal sequencing and sequence analysis

Proteins were purified from the egg white of various birds. The egg white was sonicated using Vibra cell™ sonicator (Sonics & Materials, Newtown, CT, U.S.A.) three times for 1 min on ice at power setting 4 and 50 % duty cycle. After sonication, the sample was diluted with 2 vol. of PBS [10 mM sodium phosphate buffer (pH 7.4) containing 137 mM NaCl and 3 mM KCl] and centrifuged (for 20 min, at 20 000 g, 4 °C). The soluble fraction was further purified by affinity chromatography on a 2-aminobiotin–agarose column as reported previously [36]. Eggs were obtained from farms in central Finland, and chicken avidin was from Belovo S.A. (Bastogne, Belgium).

The N-terminal sequencing of the proteins was performed with a Procise 494 A sequencer (PerkinElmer Applied Biosystems, Foster City, CA, U.S.A.).

Sequences were aligned using the ClustalX program [37]. Relationships among avidin sequences were obtained by the Neighbour Joining method [38] from a distance matrix calculated using the Poisson correction method [39] as implemented in Mega version 2.1 [40].

Biotin-binding analyses

The biotin-binding characteristics of the various avidins were studied using an IAsys optical biosensor. Binding affinities to the 2-aminobiotin surface in 50 mM borate buffer (1 M NaCl, pH 9.5) and the reversibility of binding to biotin surface in PBS (1 M NaCl) were measured as reported previously [36].

Structural analyses

The thermal stability of the avidins was studied by using an SDS/PAGE-based method [41]. Briefly, protein sample in the absence or presence of biotin (0.02 mg/ml) was diluted to a final concentration of approx. 0.2 mg/ml in 50 mM sodium carbonate buffer (pH 8.7), and proteins were acetylated by adding *N*-hydroxysuccinimidyl ester–acetic acid conjugate to a final concentration of 0.2 µg/ml. The mixture was incubated at room temperature (23 °C) for 10 min. An equal amount of SDS/PAGE sample buffer was added to the sample and it was incubated at different temperatures (25–100 °C) for 20 min. After analysis by SDS/PAGE (15 % gel), the gel was stained with Coomassie Brilliant Blue.

Sensitivity to proteinase K was studied in the absence and presence of biotin (0.02 mg/ml) as reported previously [42]. Protein sample was diluted to a final concentration of approx. 0.2 mg/ml in a buffer containing 10 mM Tris/HCl, 1.5 mM MgCl₂, 50 mM KCl and 0.1 % Triton X-100 (pH 8.8). Proteinase K (Sigma, St. Louis, MO, U.S.A.) was added to the solution (1:25, w/w) and the mixture was incubated at 37 °C. The SDS/PAGE sample buffer was added to the samples taken at different time points (0, 15, 30 and 60 min, and 16 h). The samples were then boiled and subjected to SDS/PAGE (15 % gel). The gel was again stained with Coomassie Brilliant Blue.

The pI of each avidin was determined by isoelectric focusing, as reported previously [28]. The glycosylation patterns of the various avidins were studied by treating the proteins with endo H_f glycosidase according to the manufacturer's instructions (New England Biolabs, Beverly, MA, U.S.A.). Denaturation buffer (1.1 µl) was added to the sample (10 µl, 0.5 mg/ml) and it was boiled for 10 min. Reaction buffer (1.2 µl) and endo H_f (1000 units) were added, and the sample was incubated overnight at 37 °C. The SDS/PAGE sample buffer was added and the sample was subjected to SDS/PAGE (15 % gel) followed by staining with Coomassie Brilliant Blue.

The quality of the proteins obtained as well as the molecular mass of their tetrameric forms was assayed with FPLC gel filtration using Shimadzu HPLC equipped with a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was calibrated using the gel-filtration mixture (thyroglobulin, IgG, ovalbumin, myoglobin, vitamin B₁₂; Bio-Rad Laboratories, Hercules, CA, U.S.A.) and BSA (Roche Diagnostics, Mannheim, Germany) as molecular-mass standards. Sodium phosphate buffer (50 mM, pH 7.2) with 650 mM NaCl was used as the liquid phase. Protein samples ranging between 5 and 25 µg in a volume of 10 µl were used in the analysis.

Optical spectroscopy studies

The protein samples were diluted to 0.1 mg/ml in 10 mM potassium phosphate buffer (pH 7.0), and the absorbance spectra were measured using a PerkinElmer Lambda 25 UV/visible spectrometer. The fluorescence and excitation spectra were obtained using a PerkinElmer LS50B luminescence spectrometer with 3 nm excitation and emission slit width and 120 nm/min scan speed. An excitation wavelength of 280 nm was used for

the emission measurements. Excitation spectra were measured by detecting emissions at 340 nm. The CD spectra of proteins diluted to 5 µg/ml in 100 mM sodium phosphate buffer (pH 7.4) were measured using a Jasco J-715 CD spectrophotometer equipped with a xenon lamp. A bandwidth of 1.0 nm, response time of 4 s and scanning speed of 100 nm/min were used. The mean CD spectra from four scans were analysed using the J-700 Secondary Structure Analysis estimation program (version 1.10; Jasco) using 'yang.jsr' as a reference spectra. All measurements were performed using a cuvette with a 10 mm light path at room temperature.

Differential scanning calorimetry (DSC)

The thermostability of the avidins was studied using a Calorimetry Sciences Corp. (American Fork, UT, U.S.A.) Nano II differential scanning calorimeter [31]. The reference cell was filled with the same buffer in which the sample proteins were dissolved (100 mM sodium phosphate buffer). Proteins (0.1–0.5 mg/ml) were analysed both in the absence and presence of biotin (0.004–0.02 mg/ml). All samples were degassed before they were loaded into the differential scanning calorimeter. The thermograms were recorded as a function of temperature between 25 and 130 °C at the rate of 55.6 °C/h. Data were analysed using Microcal Origin 6.0 and the software provided by Calorimetry Sciences Corp.

Immunological analyses

Avidin proteins from various bird species and streptavidin (Sigma) were compared immunologically using indirect ELISA analysis [43]. The 96-well plate was coated with various avidins (5 µg/ml) in sodium carbonate buffer (50 mM, pH 9.6) at 37 °C for 2 h, followed by washing with PBS–Tween 20 (0.05 %, v/v) and blocking with 1 % BSA in PBS (PBS–BSA).

Serum samples from 21 cancer patients exposed to chicken avidin and streptavidin during a cure cycle and from a control group of 8 persons not exposed to (strept)avidin were supplied by the Division of Nuclear Medicine, European Institute of Oncology, Milan, Italy. Sera were diluted to 1:500 in PBS–BSA and allowed to react with the various avidins at 37 °C for 2 h. Polyvalent anti-human alkaline phosphatase (AP) (Sigma) was used as a secondary antibody (2 h, 37 °C). *p*-Nitrophenyl phosphate (1 mg/ml; Sigma) was used as the signal molecule, and absorbance was measured at 405 nm by an automated ELISA reader.

In the cross-reactivity analysis of the proteins, two polyclonal rabbit anti-avidins {Poly 1, produced at the University of Oulu, Finland; Poly 2 (Avd VIII) [44]} and five mouse mAbs {Mono 1 and 2 [43], Mono 3 (Tda2), Mono 4 (Tda6) and Mono 5 (Tda9) [45]} diluted to 1:5000 in PBS–BSA were used as a primary antibody. Goat anti-rabbit IgG AP or goat anti-mouse IgG AP (Bio-Rad Laboratories) was used as a secondary antibody. Absorbance was measured as described for the serum analysis.

RESULTS

Protein purification from bird egg white

The selection of the four bird species was done on the basis of their phylogenetic relationships and the easy availability of their eggs in Finland. Their status as farmed species also makes the use of their eggs ecologically admissible. Purification yielded homogeneous proteins as judged by SDS/PAGE (Figure 1) and gel-filtration analysis (results not shown). Purification yields ranged from 0.2 to 3 mg of avidin/100 ml of egg white.

Table 1 N-terminal sequences of avidins aligned with ClustalX

Amino acids corresponding to the glycosylation site in chicken avidin are underlined. The symbols in the last line indicate the conserved amino acids. * indicates positions which have a single fully conserved residue; '·' indicates that one of the 'strong' groups NDEQ or MILV is fully conserved; '·' indicates that one of the 'weaker' groups NEQHRK or SAG is fully conserved.

Avidin	N-terminal sequence (number of amino acids)
Chicken	ARKCSLTGKWTNDLGSN MTIGAVNSRGEFTG†
Duck	AEKCVLTGKWTNDLGSX‡MTIGAVNSXGEFT- (30)
Goose	-EKCVLTGRWTNDLGSX‡MTIGAVNTSGEFTG (30)
Ostrich	-RKCDLQGLWRNELGSX‡MTLSAHDAXGAFRG (30)
Turkey	AKKCLLTGKWTNDLGSX‡MTIGAVNNR----- (26)
	* * * * * · * * * * * * * * * * · * * * * ·

† SwissProt no. P02701.

‡ The unknown amino acid residue in this place is most probably asparagine, since the only amino acids that cannot be read in protein sequencing are the modified asparagine and cysteine-acrylamide derivatives, and there was no evidence of the latter.

Figure 1 Glycosylation patterns of avidin proteins

Samples were either treated or untreated with endo H₁ glycosidase, followed by SDS/PAGE (15 % gel) and staining with Coomassie Brilliant Blue. L, Bio-Rad low-molecular-mass markers (21.5 and 14.4 kDa); arrow in a marker lane indicates the avidin band; C, chicken avidin; D, duck avidin; G, goose avidin; O, ostrich avidin; T/L, turkey avidin and low-molecular-mass marker; +, treated with enzyme.

Figure 2 Bird-avidin sequence relationships obtained using the Neighbour Joining method in Mega version 2.1

Bar represents calculated genetic distance.

N-terminal sequences and sequence analysis

N-terminal amino acids (26–30 amino acids) were sequenced for each species (Table 1). For the duck and turkey avidins, two distinct forms of protein were found in the samples. The major (longer) form represented 70 and 60 % of the total protein in the turkey and duck avidins respectively (Table 1). The minor form lacked the most distal N-terminal amino acid in both cases. These two different forms most probably reflect an alternative signal-sequence cleavage. All the avidins showed cysteine residues corresponding to chicken avidin Cys⁴, and an N-glycosylation site at Asn¹⁷ appears to be present in all four avidins, as in chicken avidin. Although only approx. 25 % of the avidin amino acid sequence was analysed, the relationships were in agreement with the phylogenetic relationships of the species studied [46]. Based on the sequenced N-termini, relationships among the avidin proteins are shown in Figure 2.

Table 2 Biotin-binding properties of avidins

Biotin-binding properties of the avidin proteins analysed in the present study by an IAsys optical biosensor. Affinities to 2-iminobiotin surface for various avidins were measured. Reversibility of binding to biotin was determined by using a biotin cuvette.

	Chicken avidin	Goose avidin	Duck avidin	Turkey avidin	Ostrich avidin
K_d (M)*	$(2.2 \pm 1.0) \times 10^{-8}$	$(7.4 \pm 5.7) \times 10^{-8}$	$(4.7 \pm 2.1) \times 10^{-8}$	$(3.6 \pm 1.2) \times 10^{-8}$	$(5.7 \pm 2.0) \times 10^{-8}$
K_d (M)†	$(3.4 \pm 2.5) \times 10^{-8}$	$(2.3 \pm 1.7) \times 10^{-8}$	$(5.7 \pm 4.3) \times 10^{-8}$	$(4.6 \pm 2.8) \times 10^{-8}$	$(8.0 \pm 4.7) \times 10^{-8}$
k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$(5.5 \pm 0.5) \times 10^4$	$(5.5 \pm 0.6) \times 10^4$	$(4.7 \pm 0.5) \times 10^4$	$(5.1 \pm 0.5) \times 10^4$	$(3.8 \pm 0.9) \times 10^4$
k_{diss} (s^{-1})	$(1.9 \pm 1.4) \times 10^{-3}$	$(1.3 \pm 0.9) \times 10^{-3}$	$(2.6 \pm 1.5) \times 10^{-3}$	$(2.3 \pm 0.8) \times 10^{-3}$	$(3.0 \pm 1.6) \times 10^{-3}$
Reversibility (%)	4 ± 2	10 ± 2	10 ± 1	8 ± 3	4 ± 3

* Values were detected from the equilibrium response data.

† Values were detected directly from the binding curves.

Table 3 Structural properties of avidins

Avidin	Quaternary structure		Heat stability				Secondary structure			
	Gel filtration		DSC		SDS/PAGE-based method		CD (calculated)		Fluorescence maximum wavelength (nm)	
	Elution time (min)	Molecular mass (kDa)	T_m (°C) (–)biotin	T_m (°C) (+)biotin	T_i (°C)* (–)biotin	T_i (°C) (+)biotin	pl	Amount of β/β -turn (%)	(–)Biotin	(+)Biotin (relative intensity)†
Chicken	29.0	60.1	85.5 ± 0.1‡	118.2 ± 0.1‡	60	95	> 9.6§	81/19	339	330 (0.6)
Duck	30.0	48.1	89.0 ± 0.1	117.6 ± 1.0	65	95	~ 9.5	100/0	343	335 (0.7)
Goose	29.4	54.3	84.8 ± 0.1	122.9 ± 0.8	65	100	> 9.6§	100/0	339	332 (0.7)
Ostrich	29.3	55.8	84.3 ± 1.5	116.3 ± 1.1	n.d.	85	~ 9	84.1/15.9	338	332 (0.9)
Turkey	29.0	60.5	79.3 ± 0.1	115.9 ± 1.4	60	95	> 9.6§	100/0	340	332 (0.6)

* Transition temperature: a temperature at which half of the total protein was in tetrameric form [41].

† Integrated intensity of the whole emission spectra relative to that without biotin.

‡ Values are from [31]. Values from DSC analysis represent the means ± S.D.

§ The pl values of the chicken, goose and turkey avidins could not be determined accurately, because they were outside the resolving capacity of the focusing gel and because the protein with the highest pl in a marker had a pl of 9.6.

|| n.d., not determined. Monomer already at room temperature.

Biotin-binding properties

In the reversibility assay, all avidins exhibited strong binding to the biotin surface similar to that of chicken avidin, as reported previously [28]. Measures of reversibility are presented in Table 2. A nitroavidin (CaptAvidin™; Molecular Probes, Eugene, OR, U.S.A.) was used as the control protein in this analysis, giving a reversibility of $65 \pm 3\%$.

Furthermore, all the avidin variants showed similar affinities towards the 2-iminobiotin surface in the IAsys interaction analysis. Measurements of the association and dissociation rate constants and dissociation constant for the various proteins are presented in Table 2.

Biochemical analyses

According to SDS/PAGE analysis, the mass of the monomer in all the avidin proteins was almost identical (approx. 16 kDa; Figure 1). Consequently, the gel-filtration analysis showed that, similar to chicken avidin, all the new avidins were tetramers. The calculated molecular masses and the elution times of the proteins are shown in Table 3.

All avidins showed temperature stability in DSC analysis quite similar to each other (Table 3). All proteins were denatured at 79–89 °C in the absence of biotin and at 116–123 °C in the presence

of biotin. In the SDS/PAGE-based thermal stability analysis, avidins from the turkey, duck and goose showed high stability, similar to that of chicken avidin. In the absence of biotin, these proteins dissociated into monomeric form at 60–65 °C. Saturation with biotin before the analysis caused extensive stabilization, and some of the protein samples remained tetrameric even at 100 °C. In contrast with the other avidins, ostrich avidin dissociated into monomers at room temperature itself in the absence of biotin. However, saturation with biotin considerably stabilized ostrich avidin, and it failed to dissociate into monomers until the temperature was increased to 80 °C. The estimated denaturation transition temperatures T_i (temperatures at which half of the sample protein was in tetrameric form and the other half was in monomeric form [41]) are shown in Table 3. It has been shown previously [47] that in the absence of biotin, proteinase K is capable of digesting chicken avidin at an exceedingly low rate and that digestion occurs only in the loop area between β -strands 3 and 4. After 16 h of digestion, approx. 25% of the treated avidin was still intact. As a holoform (saturated with biotin), chicken avidin was entirely unbroken after 16 h of proteinase K treatment. All the other bird avidins showed a similar high resistance to proteolytic activity of proteinase K (results not shown).

Treatment with endo H_f glycosidase caused increased migration in SDS/PAGE in all avidins (Figure 1). These analyses suggest that these new avidins have carbohydrate chains similar to chicken

Figure 3 CD spectra of avidin proteins

▲, chicken; ○, duck; △, goose; ●, ostrich; □, turkey.

Table 4 Cross-reactivity of various avidins

The values are means for two independent ELISA measurements of absorbance at 405 nm after 60 min (Poly 1, Poly 2 and Mono 1) or after 45 min (Mono 2–5) colour reaction. The dilution of primary antibody was 1 : 5000.

Avidin	Poly 1	Poly 2	Mono 1	Mono 2	Mono 3	Mono 4	Mono 5
Chicken	1.35	1.10	0.73	1.00	0.96	1.28	1.24
Duck	0.49	0.71	0.00	0.00	0.00	0.00	0.00
Goose	0.99	0.82	0.00	0.00	0.00	0.00	0.00
Ostrich	0.72	0.54	0.00	0.00	0.01	0.01	0.05
Turkey	1.33	1.08	0.91	1.00	1.25	1.49	1.19

avidin. The pI of ostrich avidin was lower when compared with the other avidins examined (Table 3).

All these avidins showed a tryptophan spectrum with a maximum at approx. 282 nm. Similar fluorescence emission behaviour was observed for the chicken, turkey, duck and goose avidins, since the emission maximum moved towards blue at approx. 10 nm and the emission intensity decreased after saturation with biotin. For the ostrich avidin, only a negligible decrease in emission intensity was observed (Table 3).

All the examined proteins displayed identical CD spectroscopic characteristics (Figure 3). As with chicken avidin, the strongest positive CD was found at approx. 230 nm and another weaker positive signal was detected at approx. 280 nm [8]. The measured spectra were subjected to secondary-structure analysis, which indicated entirely the presence of β -structure or β -structure with β -turns for all avidins (Table 3).

Immunological analyses

Two polyclonal antibodies raised in rabbits against chicken avidin were used in the present study. They did not recognize avidin from the goose, duck and ostrich as well as they recognized avidin from chicken and turkey. Five mAbs against chicken avidin failed completely to recognize avidin from the goose, duck and ostrich. Instead, all these antibodies recognized avidin from the turkey in an almost identical fashion to that from the chicken (Table 4).

Responses of the patient sera towards turkey and chicken avidins were similar (Pearson correlation = 0.962; $P < 0.001$). The correlation between the responses measured for the other three avidins and chicken avidin was low (Pearson correlation = 0.017–0.050). Avidin from duck and goose elicited a more or less identical immunological response with the sera (Pearson correlation = 0.766; $P < 0.001$). Streptavidin was observed to

Figure 4 ELISA for serum samples

BSA was used as a negative control. The values are means for two independent measurements of absorbance at 405 nm after 60 min colour reaction. E, patient treated with chicken avidin; C, control group.

Figure 5 Immunoreactivity of various avidins with serum samples from three selected patients

The values are means for two independent measurements of absorbance at 405 nm after 60 min colour reaction.

be a highly immunoreactive protein in this analysis (Figure 4). When the individual responses were examined, it appeared that avidins and streptavidin do not share common antigenic epitopes. Correlation between the measured responses varied from 0.05 to 0.209, the highest value occurring between streptavidin and chicken avidin. Furthermore, no clear correlation was found between the patients who recognized either avidin or streptavidin strongly (Figure 5).

DISCUSSION

In cancer treatment, avidin and streptavidin have been used in imaging and drug pretargeting for approx. 15 years [3]. However, their biochemical properties are not optimal in all cases, and immunological responses to avidin and, especially, streptavidin hamper repeated use of these proteins. In the present study, we investigated whether avidins existing naturally in four different poultry species could offer beneficial properties for pretargeting applications in the treatment of cancer or other diseases. In this respect, isolated avidins exhibited attractive properties. Their high biotin-binding ability is essential for applications in avidin-biotin technology, and their strong resistance against proteinase K suggests their ability to resist other proteinases as well. These properties make the four avidins potential candidates for use in approaches *in vivo*.

Most importantly, with the exception of turkey avidin, all the other avidins were recognized less by patient sera when compared with chicken avidin. They might therefore be used in treatments after the administration of chicken avidin and bacterial streptavidin (Figure 5). On the other hand, a group of previously exposed patients generally showed greater recognition of streptavidin when compared with avidin. This result is in line with the earlier evidence that streptavidin is a more potent human immunogen than avidin. One significant advantage of these new avidins is that none of them exhibited a tendency to form larger aggregates, as has been found to be the case with some chicken avidin and streptavidin preparations [41]. Large aggregates penetrate poorly into solid cancer [48]. Additional studies will, however, be needed to demonstrate these expectations.

Avidins from turkey and chicken, which are closely related birds [46], showed immunological and biochemical properties similar to each other. Although there are dissimilarities in the N-terminal sequence between the two proteins (17%), none of the amino acid substitutions seemed to have a significant effect on the structure and function of the proteins. Two of these four amino acid differences lie in the most distal N-termini of the proteins and are not, therefore, expected to influence the β -barrel structure and tetrameric assembly. Based on biochemical analyses and N-terminal sequence, avidins from the goose and duck are similar to each other, but they were found to differ from chicken and turkey avidins in their immunological properties.

The ostrich avidin is a more divergent protein (53% different from the chicken avidin at the N-terminus) and displayed a different immunoreactivity. Another interesting feature of ostrich avidin is its lower tetrameric stability in the absence of biotin and in the presence of SDS, much like some chicken avidin mutants [28,29,36,49]. Saturation with biotin, however, improved the stability of ostrich avidin as for other avian avidins. The weaker stability seems to be associated with the presence of denaturing agents (SDS, 2-mercaptoethanol), because in the absence of a detergent (DSC experiment) the ostrich avidin exhibited stability comparable with other avidins studied.

Despite the sequence differences at the N-termini of the proteins, some features are conserved in the avidins examined in the present study. Each protein has a cysteine residue close to its N-terminus, suggesting the formation of an intra-monomer cysteine bridge similar to that of chicken avidin. All these avidins also seem to contain an asparagine residue, with favourable neighbour residues needed for an N-linked carbohydrate side chain at the same position as in chicken avidin. According to the deglycosylation studies, no other carbohydrate attachment sites appeared to exist. With the exception of the ostrich avidin, no noteworthy differences in the spectroscopic properties of the proteins were found, suggesting that the aromatic amino acids are well conserved among the various avidins. A comparatively lower decrease in fluorescence intensity due to biotin binding for the ostrich avidin suggests the existence of a different tryptophan pattern, or that its tryptophan residues may participate in biotin binding in a way different from that of the other avidins. For example, the role of the tryptophan residues is different in avidin and streptavidin, whereas both have high affinity to biotin [50]. The complete amino acid sequence of the new avidins is needed, however, for a more detailed discussion of the functional properties.

Ligand binding to the native state of a protein will make the protein more resistant to heat-induced denaturation [31], and the degree to which the protein is made more thermostable depends on the affinity of the ligand-protein complex [31,51]. The transition midpoint of heat denaturation, T_m , of all avidins shifted approx. 28–38 °C when saturated with biotin. This result

is in agreement with the fact that all studied avidins have similar high affinities for biotin.

The properties of these new avian avidins, such as moderate sequence variation within the N-terminal amino acids and their altered immune recognition, indicate that there are some interesting differences between the structures of avidin proteins, although they all share the same functional quality, namely a strong affinity to biotin. These results also show that screening of other avidins is a potential alternative strategy for finding new candidates for medical applications *in vivo*. The four new avidins reported here exhibited promising properties useful in biopharmaceutical applications and may be valuable as alternatives to avidin and streptavidin in medical applications. The present study inspires further research into the forms of avidin in birds and other egg-laying species. The phylogenetic relationships should provide a good starting point for such efforts.

We thank Mrs Irene Helkala and Mr Paavo Niutanen for first-class technical assistance. We thank Professor Matti Vuento for help in the CD spectroscopy. We also thank Dr Kari Airenne for his critical comments on the manuscript.

REFERENCES

- Green, N. M. (1990) Avidin and streptavidin. *Methods Enzymol.* **184**, 51–67
- Wilchek, M. and Bayer, E. (1990) Introduction to avidin-biotin technology. *Methods Enzymol.* **184**, 5–13
- Hnatowich, D. J., Virzi, F. and Rusckowski, M. (1987) Investigations of avidin and biotin for imaging applications. *J. Nucl. Med.* **28**, 1294–1302
- Sakahara, H. and Saga, T. (1999) Avidin-biotin system for delivery of diagnostic agents. *Adv. Drug Deliv. Rev.* **37**, 89–101
- Oehr, P., Westermann, J. and Biersack, H. J. (1988) Streptavidin and biotin as potential tumor imaging agents. *J. Nucl. Med.* **29**, 728–729
- Paganelli, G., Malcovati, M. and Fazio, F. (1991) Monoclonal antibody pretargeting techniques for tumour localization: the avidin-biotin system. *International Workshop on Techniques for Amplification of Tumour Targeting. Nucl. Med. Commun.* **12**, 211–234
- Bickel, U., Yoshikawa, T. and Pardridge, W. M. (2001) Delivery of peptides and proteins through the blood-brain barrier. *Adv. Drug Deliv. Rev.* **46**, 247–279
- Green, N. M. (1975) Avidin. *Adv. Protein Chem.* **29**, 85–133
- Subramanian, N. and Adiga, P. R. (1997) Mapping the common antigenic determinants in avidin and streptavidin. *Biochem. Mol. Biol. Int.* **43**, 375–382
- Schechter, B., Silberman, R., Arnon, R. and Wilchek, M. (1990) Tissue distribution of avidin and streptavidin injected to mice. Effect of avidin carbohydrate, streptavidin truncation and exogenous biotin. *Eur. J. Biochem.* **189**, 327–331
- Rosebrough, S. F. (1993) Pharmacokinetics and biodistribution of radiolabeled avidin, streptavidin and biotin. *Nucl. Med. Biol.* **20**, 663–668
- Rosebrough, S. F. and Hartley, D. F. (1996) Biochemical modification of streptavidin and avidin: *in vitro* and *in vivo* analysis. *J. Nucl. Med.* **37**, 1380–1384
- Marshall, D., Pedley, R. B., Melton, R. G., Boden, J. A., Boden, R. and Begent, R. H. (1995) Galactosylated streptavidin for improved clearance of biotinylated intact and F(ab')₂ fragments of an anti-tumour antibody. *Br. J. Cancer* **71**, 18–24
- Yao, Z., Zhang, M., Sakahara, H., Nakamoto, Y., Higashi, T., Zhao, S., Sato, N., Arano, Y. and Konishi, J. (1999) The relationship of glycosylation and isoelectric point with tumor accumulation of avidin. *J. Nucl. Med.* **40**, 479–483
- Sung, C. and van Osdol, W. W. (1995) Pharmacokinetic comparison of direct antibody targeting with pretargeting protocols based on streptavidin-biotin binding. *J. Nucl. Med.* **36**, 867–876
- Stoldt, H. S., Aftab, F., Chinol, M., Paganelli, G., Luca, F., Testori, A. and Geraghty, J. G. (1997) Pretargeting strategies for radio-immunoguided tumour localization and therapy. *Eur. J. Cancer* **33**, 186–192
- Knox, S. J., Goris, M. L., Tempero, M., Weiden, P. L., Gentner, L., Breitiz, H., Adams, G. P., Axworthy, D., Gaffigan, S., Bryan, K. et al. (2000) Phase II trial of yttrium-90-DOTA-biotin pretargeted by NR-LU-10 antibody/streptavidin in patients with metastatic colon cancer. *Clin. Cancer Res.* **6**, 406–414
- Penichet, M. L., Kang, Y. S., Pardridge, W. M., Morrison, S. L. and Shin, S. U. (1999) An antibody-avidin fusion protein specific for the transferrin receptor serves as a delivery vehicle for effective brain targeting: initial applications in anti-HIV antisense drug delivery to the brain. *J. Immunol.* **163**, 4421–4426

- 19 Kassis, A. I., Jones, P. L., Matalka, K. Z. and Adelstein, S. J. (1996) Antibody-dependent signal amplification in tumor xenografts after pretreatment with biotinylated monoclonal antibody and avidin or streptavidin. *J. Nucl. Med.* **37**, 343–352
- 20 Klibanov, A. L., Martynov, A. V., Slinkin, M. A., Sakharov, I., Smirnov, M. D., Muzykantov, V. R., Danilov, S. M. and Torchilin, V. P. (1988) Blood clearance of radiolabeled antibody: enhancement by lactosamination and treatment with biotin–avidin or anti-mouse IgG antibodies. *J. Nucl. Med.* **29**, 1951–1956
- 21 Paganelli, G., Grana, C., Chinol, M., Cremonesi, M., De Cicco, C., De Braud, F., Robertson, C., Zurrida, S., Casadio, C., Zoboli, S. et al. (1999) Antibody-guided three-step therapy for high grade glioma with yttrium-90 biotin. *Eur. J. Nucl. Med.* **26**, 348–357
- 22 Grana, C., Chinol, M., Robertson, C., Mazzetta, C., Bartolomei, M., De Cicco, C., Fiorenza, M., Gatti, M., Caliceti, P. and Paganelli, G. (2002) Pretargeted adjuvant radioimmunotherapy with yttrium-90-biotin in malignant glioma patients: a pilot study. *Br. J. Cancer* **86**, 207–212
- 23 Breitz, H. B., Weiden, P. L., Vanderheyden, J. L., Appelbaum, J. W., Bjorn, M. J., Fer, M. F., Wolf, S. B., Ratliff, B. A., Seiler, C. A., Foisie, D. C. et al. (1992) Clinical experience with rhenium-186-labeled monoclonal antibodies for radioimmunotherapy: results of phase I trials. *J. Nucl. Med.* **33**, 1099–1109
- 24 Weiden, P. L. and Breitz, H. B. (2001) Pretargeted radioimmunotherapy (PRIT) for treatment of non-Hodgkin's lymphoma (NHL). *Crit. Rev. Oncol. Hematol.* **40**, 37–51
- 25 Chinol, M., Casalini, P., Maggiolo, M., Canevari, S., Omodeo, E. S., Caliceti, P., Veronese, F. M., Cremonesi, M., Chiolerio, F., Nardone, E. et al. (1998) Biochemical modifications of avidin improve pharmacokinetics and biodistribution, and reduce immunogenicity. *Br. J. Cancer* **78**, 189–197
- 26 Meyer, D. L., Schultz, J., Lin, Y., Henry, A., Sanderson, J., Jackson, J. M., Goshorn, S., Rees, A. R. and Graves, S. S. (2001) Reduced antibody response to streptavidin through site-directed mutagenesis. *Protein Sci.* **10**, 491–503
- 27 Nardone, E., Rosano, C., Santambrogio, P., Curnis, F., Corti, A., Magni, F., Siccardi, A. G., Paganelli, G., Losso, R., Aprea, B. et al. (1998) Biochemical characterization and crystal structure of a recombinant hen avidin and its acidic mutant expressed in *Escherichia coli*. *Eur. J. Biochem.* **256**, 453–460
- 28 Marttila, A., Airenne, K., Laitinen, O., Kulik, T., Bayer, E., Wilchek, M. and Kulomaa, M. (1998) Engineering of chicken avidin: a progressive series of reduced charge mutants. *FEBS Lett.* **441**, 313–317
- 29 Marttila, A. T., Laitinen, O. H., Airenne, K. J., Kulik, T., Bayer, E. A., Wilchek, M. and Kulomaa, M. S. (2000) Recombinant Neutralite avidin: a non-glycosylated, acidic mutant of chicken avidin that exhibits high affinity for biotin and low non-specific binding properties. *FEBS Lett.* **467**, 31–36
- 30 Murray, S., Maraveyas, A., Dougan, T. and Chu, A. C. (2002) Reduction of streptavidin RYDS-mediated renal adhesion by site-directed mutagenesis. *Biochim. Biophys. Acta* **1570**, 81–88
- 31 Nordlund, H. R., Laitinen, O. H., Uotila, S. T., Nyholm, T., Hytönen, V. P., Slotte, J. P. and Kulomaa, M. S. (2003) Enhancing the thermal stability of avidin. Introduction of disulfide bridges between subunit interfaces. *J. Biol. Chem.* **278**, 2479–2483
- 32 Laitinen, O. H., Nordlund, H. R., Hytönen, V. P., Uotila, S. T., Marttila, A. T., Savolainen, J., Airenne, K. J., Livnah, O., Bayer, E. A., Wilchek, M. and Kulomaa, M. S. (2003) Rational design of an active avidin monomer. *J. Biol. Chem.* **278**, 4010–4014
- 33 Hertz, R. and Sebrell, W. H. (1942) Occurrence of avidin in the oviduct and secretions of the genital tract of several species. *Science* **96**, 257
- 34 Jones, P. D. and Briggs, M. H. (1962) Distribution of avidin. *Life Sci.* **11**, 621–623
- 35 Korpela, J. K., Kulomaa, M. S., Elo, H. A. and Tuohimaa, P. J. (1981) Biotin-binding proteins in eggs of oviparous vertebrates. *Experientia* **37**, 1065–1066
- 36 Laitinen, O. H., Marttila, A. T., Airenne, K. J., Kulik, T., Livnah, O., Bayer, E. A., Wilchek, M. and Kulomaa, M. S. (2001) Biotin induces tetramerization of a recombinant monomeric avidin. A model for protein–protein interactions. *J. Biol. Chem.* **276**, 8219–8224
- 37 Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882
- 38 Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425
- 39 Nei, M. and Kumar, S. (2000) *Molecular Evolution and Phylogenetics*, Oxford University Press, New York, NY
- 40 Kumar, S., Tamura, K., Jakobsen, I. B. and Nei, M. (2001) MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245
- 41 Bayer, E. A., Ehrlich-Rogozinski, S. and Wilchek, M. (1996) Sodium dodecyl sulfate–polyacrylamide gel electrophoretic method for assessing the quaternary state and comparative thermostability of avidin and streptavidin. *Electrophoresis* **17**, 1319–1324
- 42 Laitinen, O. H., Airenne, K. J., Marttila, A. T., Kulik, T., Porkka, E., Bayer, E. A., Wilchek, M. and Kulomaa, M. S. (1999) Mutation of a critical tryptophan to lysine in avidin or streptavidin may explain why sea urchin fibropellin adopts an avidin-like domain. *FEBS Lett.* **461**, 52–58
- 43 Laitinen, O. H., Hytönen, V. P., Ahlroth, M. K., Pentikäinen, O. T., Gallagher, C., Nordlund, H. R., Ovod, V., Marttila, A. T., Porkka, E., Heino, S. et al. (2002) Chicken avidin-related proteins show altered biotin-binding and physico-chemical properties as compared with avidin. *Biochem. J.* **363**, 609–617
- 44 Kulomaa, M. S., Elo, H. A. and Tuohimaa, P. J. (1978) A radioimmunoassay for chicken avidin. Comparison with a [¹⁴C]biotin-binding method. *Biochem. J.* **175**, 685–690
- 45 Ashorn, R., Ashorn, P., Kulomaa, M., Tuohimaa, P. and Krohn, K. (1985) Production of monoclonal antibodies against avidin. *Comp. Biochem. Physiol. B* **82**, 123–127
- 46 Sibley, C. G. and Ahlquist, J. E. (1990) *Phylogeny and Classification of Birds: A Study in Molecular Evolution*, Yale University Press, New Haven, CT
- 47 Ellison, D., Hinton, J., Hubbard, S. J. and Beynon, R. J. (1995) Limited proteolysis of native proteins: the interaction between avidin and proteinase K. *Protein Sci.* **4**, 1337–1345
- 48 Zhang, M., Yao, Z., Garmestani, K., Axworthy, D. B., Zhang, Z., Mallett, R. W., Theodore, L. J., Goldman, C. K., Brechbiel, M. W., Carrasquillo, J. A. et al. (2002) Pretargeting radioimmunotherapy of a murine model of adult T-cell leukemia with the α -emitting radionuclide, bismuth 213. *Blood* **100**, 208–216
- 49 Marttila, A. T., Hytönen, V. P., Laitinen, O. H., Bayer, E. A., Wilchek, M. and Kulomaa, M. S. (2002) Mutation of important Tyr-33 residue of chicken avidin: functional and structural consequences. *Biochem. J.* **369**, 249–254
- 50 Livnah, O., Bayer, E. A., Wilchek, M. and Sussman, J. L. (1993) Three-dimensional structures of avidin and the avidin–biotin complex. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5076–5080
- 51 Brandts, J. F. and Lin, L. N. (1990) Study of strong to ultratight protein interactions using differential scanning calorimetry. *Biochemistry* **29**, 6927–6940

Received 2 October 2002/17 January 2003; accepted 31 January 2003

Published as BJ Immediate Publication 31 January 2003, DOI 10.1042/BJ20021531