The protein chicken avidin is a commonly used tool in various applications. The avidin gene belongs to a gene family that also includes seven other members known as the avidin-related genes (AVR). We report here on the extremely high thermal stability and functional characteristics of avidin-related protein AVR4/5, a member of the avidin protein family. The thermal stability characteristics of AVR4/5 were examined using a differential scanning calorimeter, microparticle analysis, and a microplate assay. Its avidin-binding properties were studied using an isothermal calorimeter and IAsys optical biosensor. According to these analyses, in the absence of biotin AVR4/5 is clearly more stable \(T_m = 107.4 \pm 0.3 \, ^\circ C\) than avidin \(T_m = 83.5 \pm 0.1 \, ^\circ C\) or bacterial streptavidin \(T_m = 75.5 \, ^\circ C\). AVR4/5 also exhibits a high affinity for biotin \(K_D \sim 3.6 \times 10^{-14} \, M\) comparable to that of avidin and streptavidin \(K_D \sim 10^{-15} \, M\). Molecular modeling and site-directed mutagenesis were used to study the molecular details behind the observed high thermostability. The results indicate that AVR4/5 and its mutants have high potential as new improved tools for applications where exceptionally high stability and tight avidin binding are needed.

Avidin from chicken and streptavidin from the bacterium *Streptomyces avidinii* are homotetramers, and both proteins are known to exhibit high binding affinity toward biotin, a small water-soluble vitamin (1). The development of avidin-biotin technology, which is based on this tight and specific interaction, has led to (strept)avidin and avidin being exploited as universal tools in a variety of fields within the life sciences. The applications of this methodology range from affinity separation and molecular probing to medical imaging and drug targeting (2).

Avidin and streptavidin are structurally analogous up-and-down \(\beta\)-barrel proteins consisting of four identical subunits (3–5). They also share an exceptional thermal stability that increases further after binding biotin. However, according to differential scanning calorimetry, the transition midpoint of heat denaturation \(T_m\) of avidin is higher than that of streptavidin in both the absence and presence of biotin (6). Recently, we have shown that avidins isolated from other avian species also have thermal stability comparable to that of chicken avidin (7).

The stability and assembly of the quaternary structure of both avidin and streptavidin have been previously modified by site-directed mutagenesis. In some studies the (strept)avidin tetramers have been either destabilized or the tetramers have been totally disintegrated into monomers or into dimers (8–13), whereas in some studies the stability of the tetramer has been improved (14–16). In these studies the enhancements in stability have been achieved by introducing intermonomeric disulfide bridges between the subunits of the avidin or streptavidin tetramers.

Protein stability is an outcome of many different factors. Some general “rules” have been established that explain the differences in stability between structurally related high-stability and low-stability proteins (17, 18). It has been shown that the flexibility of loop structures plays an important role in determining the stability of the protein (19). In this respect, it has been suggested that reduced loop length (20–22) and the presence of Gly→Pro substitutions in loops that reduce flexibility (23, 24) are reasons for the differences in stability found among homologous proteins. Another important factor is protein glycosylation, which increases the stability of some proteins (23, 25–27). For avidin, however, no such function for the attached carbohydrate has been found (27).

Chicken avidin is encoded by a gene that belongs to a gene family containing also seven avidin related genes (AVRs) (28, 29). The number of AVR genes seems to vary between individuals, but seven different genes, AVR1-AVR7, have been cloned and sequenced. Two of them, AVR4 and AVR5, are identical in their coding sequence, whereas the others are 94–99% identical to each other. Because it is not known whether the AVR genes are expressed as proteins in the chicken, we cloned recently their cDNAs and produced the recombinant proteins using a baculovirus-insect cell expression system (30). The aim of the work was to study whether biochemical and functional properties of the proteins encoded by AVRs would differ from those of avidin. It was revealed that the physico-chemical properties as well as the avidin-binding properties of the expressed AVR proteins were different when compared with those of avidin and each other. The only AVR that exhibited an affinity to biotin and 2-iminobiotin comparable to that of avidin was...
AVR4/5. Nonetheless, AVR4/5 showed other interesting differences, i.e. an “extra” cysteine residue in every subunit and a different pattern of glycosylation, when compared with avidin.

Avidin is not cleaved by trypsin or Pronase, and the avidin-biotin complex is resistant even to proteolysis by the enzymes of the digestive tract (1). Proteinase K cleaved avidin only in one site, and the avidin-biotin complex was resistant to its proteolytic activity (31).

In the present study, we performed a more detailed examination of the structural and stability properties of the AVR4/5 protein using comprehensive biochemical and mutational analyses. Interestingly, we found that AVR4/5 displays the highest heat stability characteristics of all biotin-binding proteins so far characterized. AVR4/5 exhibited a $T_m$ that evidently exceeds the boiling point of water even in the absence of the ligand. The hypothetical molecular details behind the differences in stability between avidin and AVR4/5 were studied by molecular modeling. Due to its high thermal stability, AVR4/5 or some of its derivatives may prove extremely useful in applications where high heat resistance is required.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis of AVR4/5 and the Purification of Recombinant Proteins**—Site-directed mutagenesis of AVR4/5 cDNA (30) was performed by using the QuikChange (Stratagene, La Jolla, CA) method. Recombinant baculoviruses producing AVR4/5 forms were generated with the Bac-To-Bac™ system (Invitrogen). Proteins were produced in baculovirus-infected Sf9 insect cells as reported previously (30) and purified with 2-iminobiotin affinity chromatography as previously described (10). Avidin concentrations were calculated from the absorbance at 280 nm using an extinction coefficient of 24280 M$^{-1}$ cm$^{-1}$ for avidin, 26960 M$^{-1}$ cm$^{-1}$ for AVR4/5 and AVR4/5(N43E) and 26840 M$^{-1}$ cm$^{-1}$ for AVR4/5(C122S) (1).

**Glycosylation Analysis**—The glycosylation patterns of the proteins were studied by treating the proteins with endoglycosidase H, glycosidase (New England Biolabs, MA) as described in Ref. 30.

**Proteolysis Assay**—Resistance of AVR4/5 and AVR4/5(C122S) toward proteolytic activity of proteinase K was determined as previously described (30).

**Gel Filtration Analysis**—The oligomeric state of the AVR4/5 and its mutant forms were assayed by FPLC gel filtration as described in Ref. 7. Intertetrameric disulphide bridges were reduced by adding 2-mercaptoethanol to a final concentration of 10% (v/v) (at 37°C for one hour). Non-reducing SDS-PAGE analysis was performed as described in Laitinen et al. (30).

**Interaction Analyses**—The reversibility of biotin binding and the determination of binding affinities to a 2-iminobiotin surface were measured using an IAsys optical biosensor as previously reported (30). Avidin and AVR4/5(C122S) were also analyzed in 50 mM sodium acetate, pH 4.0 (100 mM NaCl). Furthermore, AVR4/5 was analyzed in the presence of 2-mercaptoethanol (0.4% v/v). The samples were analyzed both in the absence and presence of a 3-fold molar excess of biotin per subunit. The change in the heat capacity upon unfolding ($\Delta C_p$) was measured for avidin and AVR4/5(C122S) using DSC data from measurements at pH 4.0 and 7.0. The $\Delta C_p$ was then obtained from the slope of the plots of $\Delta H$ against $T_m$ (32).

**Isothermal Titration Calorimetry (ITC)**—Binding enthalpies ($\Delta H$) were measured with an ITC-4200 (Calorimetric Sciences Corporation, Provo, UT). The measurements were performed at 25 and 37°C with 1.0 ml of protein solution (20–25 $\mu$M) under constant stirring. Biotin (0.395 mM) was titrated into the sample cell in 10-$\mu$l aliquots. Analysis of the data was performed using TITRATION BINDWORKS 3.0.69 (Applied Thermodynamics, Hunt Valley, MD). The $\Delta C_p$ was calculated from the slope of the plots of $\Delta H$ against temperature ($T$).
**Table 1**

Structural properties of avidins

| Protein          | Gel filtration | Elution time (s) | Molecular mass (kDa) | Tm (°C) | ΔH° (kJ/mol) | ΔCp (J/K mol) |
|------------------|----------------|------------------|----------------------|---------|-------------|---------------|
| Chicken avidin   |                |                  |                      |         |             |               |
|                  |                | 28.5             | 61.4                 | 83.5 ± 0.1 | 17.0 ± 0.7 | 33.5 ± 2.8    |
| AVR4/5           |                | 28.9             | 55.1                 | 107.4 ± 0.3 | 124.7 ± 0.5 | 17.3 ± 1.0    |
|                  |                | 25.6             | 121.6                |          |             |               |
|                  |                | 23.5             | 200.5                |          |             |               |
| AVR4/5 (N43E)    |                | 29.5             | 48.3                 | 104.9 ± 0.3 | n.d.†       | n.d.†         |
|                  |                | 26.0             | 110.3                |          |             |               |
|                  |                | 23.9             | 188.2                |          |             |               |
| AVR4/5 (C122S)   |                | 29.0             | 54.8                 | 106.4 ± 0.8 | 125.4 ± 0.8 | 19.5 ± 0.4    |
| AVR4/5 + 2-mercaptoethanol | | 28.9 | 55.1 | 106.5 ± 0.3 | 125.2 ± 0.1 | 18.7 ± 0.3 |

† ΔTm is the change in Tm upon addition of a three-fold molar excess of biotin.

‡ ΔH value obtained from sample without biotin. The value could not be determined accurately from samples saturated with biotin because the Tm values were too close to the temperature limit of the DSC equipment.

Three protein forms with lowest molecular masses are indicated.

Three not measured.

Calculation of Binding Constants—The binding constants were calculated by combining DSC and ITC data as previously described (33) and as follows in Equations 1 and 2,

\[ K_a(T_m) = \frac{[\exp(-\Delta H/T_m)/R(1/T_m - 1/T_0)]}{[\exp(-\Delta H_0/T_0)/R(1/T_0 - 1/T_1)]} \]  

(Eq. 1)

\[ K_a(T) = K_a(T_m)\exp[-\Delta H_0/(R(1/T - 1/T_0))] \] (Eq. 2)

where \( K_a(T_m) \) is the binding constant at \( T_m \), \( \Delta H(T_m) \) is the change in enthalpy upon unfolding without the ligand, \( T_m \) is the unfolding temperature with bound ligand, \( T_0 \) is the unfolding temperature without bound ligand, \( \Delta C_p \) is the change in heat capacity upon unfolding, \( U(T_m) \) is the free ligand concentration at \( T_m \), \( K_a(T) \) is the binding constant at temperature \( T \), \( \Delta H_0(T) \) is the binding enthalpy at temperature \( T \), \( \Delta C_p \), is the heat capacity change upon ligand binding, and \( R \) is the gas constant. All values were obtained from experimental data. \( \Delta G \) was then calculated from \( -RT \ln K_a \) and \( \Delta S \) was derived from \( \Delta G = \Delta H - T \Delta S \).

Microplate Assay—The avidin-binding activity of the proteins after heat treatment at 99.9 °C was studied with a microtiter plate assay as previously reported (16).

Structural Modeling and Sequence Analysis—The three-dimensional structure of avidin in complex with biotin (Protein Data Bank code 1avd (5)) was used as the basis for modeling the structure of AVR4/5. The sequence alignment of avidin and AVR4/5 was done using MALIGN (34) in BODIL using a structure-based sequence comparison matrix (35) with gap-penalty 40.²

HOMODGE in BODIL was used to construct a three-dimensional model by keeping the side-chain conformations of all identical residues fixed and by maintaining torsion angles of similar residues in the alignment. The intramolecular interactions of the amino acids that are different from those in the template structure were optimized by using an amino acid side-chain rotamer library (36).

Microparticle Analysis—Microparticles (Bangs Laboratories, Fishers, IN) (1 × 10⁶ particles) were washed twice with water and suspended in 200 μL PB buffer (50 mM NaH₂PO₄-HCl, pH 3.0). Biotin-binding protein (1 or 3 mg/ml) in 50 μl of 50 mM sodium-acetate, pH 4.0) was added while mixing with a Vortex. The suspension was incubated under continuous shaking at 23 °C for 1 h. Particles were washed with PB and then with 50 mM MES buffer (pH 5.5) and suspended in 200 μl of MES buffer. 200 ml of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce, Rockford, IL) (1 mg/ml) in MES buffer was added, and the suspension was incubated as above. Particles were then washed once with MES buffer, four times with AB buffer (50 mM Tris-HCl, 10 mM NaNO₃, 1 mM EDTA, 0.1% Tween 20, pH 8.0) and suspended in 100 μL of AB. Particle concentration was determined by a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA). Streptavidin-coated microspheres (3.12 μm) were purchased from Bangs Laboratories.

Microparticles (5 × 10⁶ particles) suspended in the AB buffer were aliquoted into four PCR tubes (i-iv). BF523-biotin (ArcDia Ltd., Turku, Finland) was added to a final concentration of 20 nM in aliquots i and ii, which were incubated under continuous shaking for one hour. Aliquots ii and iv were then incubated at 95 °C, whereas aliquots i and iii were kept at room temperature for 30 min. BF523-biotin (20 nM) was added to aliquots iii and iv after thermal treatment. All aliquots were incubated in a shaker at 22 °C and 1100 rpm for 2 h and transferred as three replicate samples to a 384-well plate. The samples were measured with the ArcDia TPX PlateReader (37). The negative control for the assay was prepared by saturation of the coated particles with an excess of unlabeled biotin (100 pmol/1 × 10⁶ particles) prior to incubation with BF523-biotin.

The fluorescence emission efficiency of the BF523-biotin conjugate (290 nm) was determined before and after the incubation at 95 °C for 30 min. The 14.1% decrease in probe fluorescence obtained was used throughout the analyses to correct the results.

**RESULTS**

Mutagenesis and Production of AVR4/5—On the basis of the sequence alignment of AVR4/5 and avidin (Fig. 1), Asp-43 of AVR4/5 was mutated to glutamate to avoid possible glycosylation at that position. Cys-122 in AVR4/5 was converted to serine to eliminate the formation of extraneous disulfide bridges in this protein. Serine was chosen on account of the sequence differences that AVR4/5 and avidin show at their C termini. Serine is a polar residue similar in size to cysteine. The resulting mutants were designated as AVR4/5(N43E) and AVR4/5(C122S) and they were produced as described in Ref. 30.
Table II

Thermodynamic parameters for biotin binding to AVR4/5 and avidin

All values are the averages of at least three different experiments ± S.D.

| Sample          | $K_a$ (25°C) | $K_d$ (25°C) | $\Delta H$ (25°C) | $\Delta H$ (37°C) | $\Delta C_p$ | $\Delta G^a$ | $\Delta S^a$ |
|-----------------|--------------|--------------|-------------------|-------------------|-------------|-------------|-------------|
| Avidin          | 1.6 ± 0.9 × 10⁻⁷ | 1.4 ± 0.4 × 10⁻⁷ | 1.1 ± 0.4 × 10⁻⁷ | 1.4 ± 0.6 × 10⁻⁷ | 2.2 ± 1.0 × 10⁻⁸ | 3.4 ± 2.5 × 10⁻⁸ |
| AVR4/5(C122S)  | 2.3 ± 2.2 × 10⁻⁷ | 1.5 ± 1.2 × 10⁻⁷ | 1.3 ± 0.3 × 10⁻⁷ | 1.8 ± 0.8 × 10⁻⁷ | 5.5 ± 0.5 × 10⁻⁸ | 1.9 ± 1.4 × 10⁻³ |
| Reversibility (%) | 5 ± 3         | 5 ± 3         | 3 ± 2             | 4 ± 2             |             |             |

* The binding constant $K_a$ calculated from DSC and ITC results according to Equations 1 and 2.

Table III

Biotin-binding properties of proteins determined by optical biosensor

Measured affinities to 2-iminobiotin surface for avidin and different AVR4/5s. Reversibility of binding to biotin was determined by using a biotin cuvette.

| Sample          | $K_a$ (M⁻¹) | $K_d$ (M) | $\Delta H$ (mJ/mol) | $\Delta C_p$ (kcal/mol) | $\Delta G^a$ (kcal/mol) | $\Delta S^a$ (cal/mol K) |
|-----------------|-------------|-----------|---------------------|-------------------------|-------------------------|----------------------------|
| Avidin          | 1.6 ± 0.9 × 10⁻⁷ | 1.4 ± 0.4 × 10⁻⁷ | 1.1 ± 0.4 × 10⁻⁷ | 2.2 ± 1.0 × 10⁻⁸ | 3.4 ± 2.5 × 10⁻⁸ | 1.9 ± 1.4 × 10⁻³ |
| AVR4/5(C122S)  | 2.3 ± 2.2 × 10⁻⁷ | 1.5 ± 1.2 × 10⁻⁷ | 1.3 ± 0.3 × 10⁻⁷ | 1.8 ± 0.8 × 10⁻⁷ | 5.5 ± 0.5 × 10⁻⁸ | 1.9 ± 1.4 × 10⁻³ |

Because AVR4/5(C122S) was found to bind biotin as AVR4/5 and to oligomerize like avidin, it was selected for ITC measurements. As these proteins have a very high affinity for biotin, no binding constant could be calculated from the ITC experiments alone. However, because titration binds all the ligands, the binding enthalpy could be calculated from the heat released upon the injection of biotin. The ITC experiments, performed at 25 and 37°C, showed that the values of both $\Delta H$ and $\Delta C_p$ were similar for biotin binding to AVR4/5(C122S) and avidin (Table II). Both the binding enthalpy for biotin binding and the $\Delta C_p$ were similar to previously reported values (39).

Fig. 4. Microplate assay. AVR4/5 shows superior stability against boiling as compared with avidin as well as to high-stability AVD-ci (average, n = 4).

The estimated binding constant $K_a$ calculated from a combination of ITC and DSC data showed that the binding of biotin to AVR4/5(C122S) ($K_a = 2.8 \times 10^{13} \text{ M}^{-1}$) was almost two orders of magnitude lower than for avidin ($K_a = 9.3 \times 10^{15} \text{ M}^{-1}$). The difference in how avidin binds to AVR4/5(C122S) and avidin may be interpreted from the thermodynamic data in Table II. Because the value of $\Delta H$ is rather similar for biotin binding to both AVR4/5(C122S) and avidin, the difference in $\Delta G$ is expected to be mainly due to an entropic contribution.

The Microplate Assay—The microplate assay showed that AVR4/5 and its mutants resist boiling and remain in an active form significantly longer than chicken avidin or even a stabilized avidin, AVD-ci, with additional intermonomeric disulfide bonds (16). AVR4/5(C122S) showed the greatest stability, because about 30% of the initial binding activity remained present after boiling for 32 min (Fig. 4).

Computational Analyses—Molecular modeling and sequence
analysis identified amino acid substitutions between avidin and AVR4/5 that might explain the observed differences in the stability and functional properties of AVR4/5. For example, Gly-21 and Gly-107 are found in loops 2 and 7 in avidin, but they are not present in AVR4/5. On the other hand, loop 3 of AVR4/5 contains the Pro-41-Gly-42 sequence that is not present in avidin (Fig. 1).

In Fig. 5, sequence differences between avidin and AVR4/5 are highlighted for residues where the side chains are involved in interactions at the subunit interfaces. The largest interface occurs between subunits 1 and 4 (and 2–3), involving 31 amino acid side-chains from both subunits. This interface shows only four sequence differences. Tyr-55 and Ile-56 in avidin correspond to a deletion in AVR4/5 at a loop located between β-strands 4 and 5. In avidin, the hydrophobic side-chain of Ile-56 of subunit 1 is surrounded by the polar main-chain oxygen atoms of Trp-70, Lys-71, and Ser-73 from subunit 4 (Fig. 5A). In AVR4/5, it is likely that the deletion allows favorable interactions between the positively charged side-chain of Arg-56 and the polar main-chain oxygen atoms of Trp-68, Asn-69, and Ser-71 (Fig. 5B).

In the avidin structure, a hydrophobic interaction is formed between Ile-117 from subunit 1 and Ile-117 from subunit 3 (or subunits 2 and 4). In avidin, Ile-117 is surrounded by several water molecules, reflecting the neighboring polar environment (Fig. 5C). In AVR4/5 (Fig. 5D), Tyr-115 can form an additional favorable interaction with Tyr-115 from the other subunit. Tyr-115 in AVR4/5 would most likely displace two water molecules and interact with the remaining water molecules and surrounding amino acid residues.

Asp-39 in AVR4/5 corresponds to alanine in avidin being located at the interface between subunits 1 and 2. The side chain of alanine in the avidin structure is unable to form any intramolecular interactions, but only lines the ligand-binding pocket (Fig. 6A). In contrast, Asp-39 in AVR4/5 most likely forms a salt bridge with Arg-112 from the same subunit, which could restrict the size of the entrance to the ligand-binding cavity (Fig. 6B). In addition, this aspartate may interact with the positively charged end of the Lys-109 side chain from a neighboring monomer.

**Microparticle Assay**—Characteristics of AVR4/5 and AVR4/5(C122S) were studied by coating plastic microparticles with proteins. The capacity of AVR4/5-coated particles was notably higher than that of commercial streptavidin particles and particles coated with wild type avidin and high-stability (Tm = 98.6 °C) AVD-ci with two intersubunit disulfide bonds (16) (Table IV, i and iii). The specificity of BF523-biotin binding to protein-coated particles was confirmed by saturating the particles with unlabeled biotin prior to analysis.

When particles coated with biotin-binding proteins were heat-treated, the AVR4/5 forms, compared with other proteins,
showed excellent heat resistance (Table IV, iv). AVR4/5 and AVR4/5(C122S) showed 93% and 75% of the original signal after heating. AVR-ci showed higher durability (35%) than wild type avidin (13%). When the biotin-conjugate was added before heat-treatment (ii), all the particles showed excellent heat treatment compared to those obtained from particles without treatment.

### DISCUSSION

The chicken avidin gene is a member of a gene family containing at least eight members (28, 29). However, the role of other members of the family, known as avidin-related genes (AVRs) and the AVR-proteins that they encode, is not known. AVR4/5 is the closest relative to the avidin gene in the family, and its chromosomal location is closest to the avidin gene (40). In a preceding study it was noticed that both the biotin-binding and physico-chemical characteristics varied between different AVRs and avidin (30). The most obvious structural differences would arise from the diverse patterns of glycosylation, and the extraneous cysteine residue found in most AVRs that is not present in avidin. In addition, residues in contact at the subunit interfaces of the model structure showed significant differences when compared with the structure of avidin. However, according to the stability analysis by SDS-PAGE (41), these differences at the interfaces did not reduce the stability of the AVRs. To address these issues more thoroughly, we have in the current study concentrated on studies of the characteristics of AVR4/5.

The most surprising observation of the present study was the superior heat stability of AVR4/5 and its mutants when compared with avidin or streptavidin (6), as well as to the thermally improved forms of avidin (16). In the latter case, the improvements in stability were achieved by introducing disulfide bridges between avidin subunits. In contrast, the disulfide bridge due to the extraneous Cys-122 of AVR4/5 could not be connected to the enhanced stability, because AVR4/5(C122S) was observed to be even more stable than AVR4/5. According to ITC, DSC, and optical biosensor data, the biotin and 2-iminobiotin binding properties of AVR4/5 resemble those of avidin. The measured affinity toward biotin \( K_d \approx 3.5 \times 10^{-14} M \) is weaker than that for avidin, but this interaction nonetheless remains one of the tightest measured for ligand-protein interactions. In fact it resembles values measured between avidin and bacterial streptavidin \( K_d \approx 4.0 \times 10^{-14} M \) (42). The thermodynamic explanation for the weaker affinity of AVR4/5 toward biotin (as compared with avidin) seems to lie in the change in entropy on binding.

The modeling results suggest that possible reasons for the improved thermal stability of AVR4/5 include both a more stable tertiary \( \beta \)-barrel structure as well as the more favorable subunit interactions of the tetramer. The structure of avidin may be more flexible and in a more open conformation, thereby facilitating the entry of biotin into the binding pocket, than the potentially more rigid AVR4/5 barrel. This might also explain the higher affinity of avidin toward biotin and 2-iminobiotin. The potential salt bridge between Asp-39 and Arg-112 in AVR4/5 may contribute to the slower rate of association observed in the case of AVR4/5 for 2-iminobiotin, and it may also stabilize the monomer structure. Furthermore, the interaction of Asp-39 with the positively charged Lys-109 from the neighboring subunit may enhance the stability of the tetramer.

One important contributory factor to the enhanced stability of AVR4/5 is probably connected to the deletion of two residues in loop 4 of the protein, corresponding to residues Thr-55 and Ile-56 in avidin. This deletion may eliminate the unfavorable interaction seen in avidin. In general, comparison of the crystal structure of related proteins have shown that shorter loops are less mobile and presumably more stable (20–22). In addition, the Ser-41 to proline conversion in loop 3 may add rigidity to the secondary and tertiary structure of AVR4/5. The replacement in AVR4/5 of glycine residues, present in avidin, may have a further stabilizing effect, because glycine has more freedom to adopt torsion angles not permitted in other amino acids (23, 24).

Finally, the replacement of Ile-117 in avidin by tyrosine in AVR4/5 should increase the stability of the 1–3 (and 2–4) dimer interface in comparison with avidin. Furthermore, the stabilizing effect might also extend to subunits 2 and 4 (1 and 3), which are in close proximity to the 1–3 (2–4) dimer interface (Fig. 5D). Additional mutational and structural analyses are, however, needed to validate the accuracy of these proposals.

Although the predicted disulfide bridges seemed to have no effect on the thermal stability of AVR4/5, they had a clear influence on the overall structure of the protein. In contrast to the previous hypothesis (30), the FPLC data suggested that these disulfide bridges connect AVR4/5 tetramers together, thus forming larger oligo-tetrameric structures instead of linking the monomers within the same tetramer (Fig. 2). Multimerization has direct consequences on the possible applications in which AVR4/5 could be exploited. In some cases where high thermal stability connected to a clearly defined molecular mass is needed, then AVR4/5(C122S) could be the best option. In other circumstances, for example where high biotin-binding capacity together with high thermostability is required (43), native AVR4/5 might be the most suitable candidate (Table IV).

AVR4/5 contains three potential N-glycolysation sites. In a previous study (30) it seemed that AVR4/5 was heavily glycosylated, but it was impossible to judge if all the possible sites were utilized. Mutational analysis showed that glycosylaion occurs at least at Asn-43 (Fig. 3). However, the glycosylation of Asn-43 had no marked influence on the biotin binding, thermal

### Table IV

**Microparticle assay of biotin-binding proteins**

| Particle type | Pre-loaded (i) | Pre-loaded, thermally treated (ii) | Fluorescence left after thermal treatment | Post-loaded (iii) | Post-loaded, thermally treated (iv) | Fluorescence left after thermal treatment |
|---------------|----------------|-----------------------------------|-----------------------------------------|------------------|------------------------------------|-----------------------------------------|
| Streptavidin  | 100            | 57                                | 57                                      | 106              | 22                                 | 21                                      |
| AVR4/5        | 192            | 239                               | 124                                     | 145              | 28                                 | 21                                      |
| AVR4/5 (3 mg/ml)* | 417          | 359                               | 56                                      | 437              | 270                                | 93                                      |
| AVR4/5(C122S) | 61             | 92                                | 150                                     | 65               | 49                                 | 75                                      |
| AVD           | 41             | 16                                | 38                                      | 47               | 6                                  | 13                                      |
| AVD (3 mg/ml)* | 54             | 20                                | 37                                      | 57               | 5                                  | 9                                       |
| AVR-ci        | 72             | 45                                | 62                                      | 82               | 29                                 | 35                                      |

* A higher protein concentration was used in the coating procedure.
stability or structural properties of AVR4/5. A portion of the resultant protein AVR4/5(N43E) appeared to be even more glycosylated than avidin, suggesting that both remaining glycosylation sites (Asn-69 and Asn-117) could be utilized at least to some extent.

Although the present study does not unequivocally prove that AVR4/5 is an ex officio member of the chicken proteme, it does provide circumstantial evidence supporting this. High thermal stability per se is not a requirement in the chicken, but it may well reflect a greater ability to resist other environmental factors. Whatever the situation is in the chicken we can, nevertheless, exploit this protein in applications that are performed under harsher conditions than native avidin can withstand.

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